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ACID FORMATION IN FROZEN AND THAWED ARBACIA PUNCTULATA EGGS AND ITS POSSIBLE BEARING ON THE PROBLEM OF ACTIVATION

JOHN RUNNSTRÖM [1]

(From the Institute of Experimental Zoölogy, Stockholm, Sweden, and the Marine Biological Laboratory, Woods Hole)

According to observations reported by E. B. Harvey (1930) and the writer (Runnström, 1930) the sea urchin egg can be fertilized under anaerobic conditions. The further development, however, even the fusion of the pronuclei, is blocked. The writer (Runnström, 1930, 1933) furthermore stated that an acid is formed following aerobic as well as anaerobic fertilization of the *Paracentrotus* egg. The quantity of acid formed was found by Warburg's manometric methods to be equal to about 150 cu. mm. per cc. of egg substance. Under anaerobic conditions the acid formation seems to be 20–25 per cent higher. Acid formation also takes place after treatment of the eggs with hypertonic solutions. Higher values were found under anaerobic than under aerobic conditions. Acid formation is also increased in the *Paracentrotus* egg with increasing concentrations of the hypertonic solutions. The acid formation does not correspond to an increase in lactic acid formation nor to an increase of the content of inorganic phosphate (Runnström, 1933). Örström (1935) found that the formaline titration did not reveal any increase in the amino-nitrogen content following fertilization of the *Paracentrotus* egg. The same author (1935) also found that ammonia is formed at a slow rate in the unfertilized *Paracentrotus* eggs. Upon activation by sperm or by a hypertonic solution there is a marked increase of ammonia production, corresponding to about 0.25 cc. n/100 NH_3 per 1 cc. of egg substance. After the outburst following activation ammonia production stops in the fertilized egg. In spite of the formation of ammonia in the activation process the egg suspension gets more acid due to the simultaneous formation of an acid.[2]

[1] Fellow of The Rockefeller Foundation, 1933–34.

[2] In the course of further experiments Örström arrived at the conclusion that not free ammonia but a compound containing ammonia is formed upon fertilization. Ammonia is split off from this compound on applying the Parnus procedure for ammonia estimation.

In a previous paper (Runnström, 1933) it was stated that a breaking up of the *Paracentrotus* eggs in distilled water is accompanied by a strong acid formation. The further study of the acid formation in the broken up eggs seemed of interest in view of its possible bearing on the activation process. The observations were carried out at the Marine Biological Laboratory, Woods Hole, with the eggs of *Arbacia punctulata*. The work was also regarded as a preparation for further attempts towards the chemical identification of the acid formed.

Dr. Alfred Mirsky suggested to the writer a method of breaking up the eggs which is more appropriate than the treatment with distilled water and has great advantages also for the determination of acid formation. The eggs are frozen at about —80° in carbon dioxide snow and ether. After a longer or shorter time the eggs are thawed at room temperature. Here the influence of different substances on the remaining metabolism can be tried without interference by the semipermeability of the egg surface. The acid formation was measured by the Warburg manometric method. Three cc. of egg suspension were transferred to each vessel; one side-arm contained 0.3 cc. 2–n H_2SO_4. The vessels were carefully stoppered and immersed into the mixture of ether and dry snow. After some time the vessels were removed from this mixture and the suspension thawed at room temperature. The vessels were joined to the manometers after one hour of thawing. After establishment of the temperature and gas equilibrium, sulfuric acid was tipped into the main compartment of the vessel. The manometric readings give the content of combined carbonic acid in the thawed egg suspension (B II). The combined carbonic acid was determined in the same way in a non-frozen portion of the egg suspension (B I). The difference between the two determinations of the combined carbonic acid $\Delta B = B\,I - B\,II$ is proportional to the acid formation. Samples of sea water were treated in the same way as the egg suspensions but no significant differences were found in this case. The value obtained for ΔB should be multiplied by a "retention factor" which, however, has not been determined for this material. The values given below are uncorrected and it is to be remembered that they are throughout lower than the real values.

In one experiment, July 25, 1935, B I was found to be 127 and B II 21 cu. mm., ΔB is then 106 cu. mm. The concentration of the suspension was 7.5 vol. per cent; this means about 470 cu. mm. acid per 1 cc. egg substance. In a parallel experiment to this the egg suspension contained 0.035 m sodium monoiodoacetate. In this case B I was 125 cu. mm., B II 20.5 cu. mm., ΔB is thus 104.5 cu. mm. The agreement between the two ΔB values shows that iodoacetate has no influence on the acid formation.

In one experiment, July 26, 1934, B I was 131 cu. mm. and B II 19.5 cu. mm. ΔB is thus 116.5 cu. mm. As the suspension was 8.6 vol. per cent this means 450 cu. mm. per 1 cc. of egg substance. In a parallel experiment the egg suspension contained 0.0004 per cent $CuCl_2$. B I was 132, B II 13 cu. mm., $\Delta B = 119$ cu. mm. It is evident that copper does not inhibit the acid formation.

In the same way it has been shown that 0.06 m sodium fluoride has no influence on the acid formation in the thawing eggs. The addition of 0.008 per cent pyocyanine was likewise without any effect.

In one experiment, July 27, 1934, the influence of hexosemonophosphate was tested. To 3 cc. egg suspension 0.15 cc. 0.25 M hexosemonophosphate was added (Robison ester). ΔB was here 90 cu. mm., while it was 88 cu. mm. without addition of hexosemonophosphate. In a parallel experiment with hexosemonophosphate the egg suspension contained also 0.02 M iodoacetate. ΔB was here 98 cu. mm. The suspension was 16 vol. per cent. The figures tend to show that hexosephosphate has no essential influence on the acid formation. The retention factor may be somewhat higher for the mixture containing hexosephosphate, cf. the analogous case with the eggs cytolyzed in distilled water (Runnström, 1933, p. 276). If the acid formation were a glycolysis enhanced by addition of hexosephosphate, the acid formation should be decreased by addition of iodoacetate which inhibits the oxidoreductions involved in glycolysis. Even in suspensions with hexosephosphate added the acid formation is the same either with or without iodoacetate.

An acid formation took place also in thawing fertilized eggs. This is about equal to that found in unfertilized eggs. In one experiment (August 1, 1934) ΔB was found to be 117 cu. mm. for unfertilized and 107.5 cu. mm. for fertilized eggs. The suspension was 10 vol. per cent.

In some of the later experiments the suspension of thawed eggs was centrifuged, the solid parts collected as a pulp on the bottom of the tubes, separated from a colored fluid. The combined carbonic acid was determined in the fluid part. Very low values were now found as compared with the values obtained with the uncentrifuged thawed eggs.

In one experiment (August 18, 1934) the eggs were frozen and kept so for two days, then thawed. B I was 278 cu. mm., B II 144 cu. mm., ΔB is then 134 cu. mm. A portion of the thawed eggs was centrifuged. After this B II was found to have the value of 48 cu. mm. and ΔB is thus 230 cu. mm. This curious fact may be explained by assuming that some proteins centrifuged down in the quoted experiment acquire a CO_2-binding capacity during the acid formation which may

bring them to the acid side of their isoelectric point. Since, in every experiment, the different samples to be compared were treated in exactly the same manner, the reported result will not annihilate the conclusions drawn above from experiments with uncentrifuged thawed eggs. As pointed out already, the figures given have only relative value.

The thawed eggs show an oxygen uptake, which is very much enhanced by the addition of pyocyanine or methylene blue. The oxygen uptake is still more increased on addition of hexosephosphate (cf. Runnström, 1935); 0.03 m iodoacetate or 0.06 m fluoride inhibit the respiration enhanced by hexosephosphate 30–35 per cent.

Unfertilized eggs were transferred into the fluid extract remaining after centrifuging off the solid particles from thawed eggs. After some few minutes a number of the eggs are surrounded by an easily visible, distinct jelly. Probably the visible jelly is a coagulation product of the jelly surrounding the egg. This jelly in general can only be demonstrated by the addition of certain stains or India ink. The neutralized extract exhibited the same influence on the jelly. Heating the extracts for 10 minutes to 70° does not remove the action on the jelly. In the course of the present work an acid formation amounting to 150–170 cu. mm.[3] per 1 cc. of egg substance was found to follow fertilization also in *Arbacia*. A similar acid production takes place upon aerobic treatment of the unfertilized *Arbacia* eggs with a stronger hypertonic solution according to Just (1922). The question arises as to whether the acid formation in the thawing eggs has the same chemical basis as the acid formation following the activation of the egg. This cannot be decided as yet but seems very likely. In the *Paracentrotus* egg a gradual increase of acid formation was found with increasing concentration of the hypertonic solution (cf. above). Finally concentrations are reached in which the eggs are cytolyzed. It must be pointed out that after cytolysis the acid formation stops, both in the eggs treated by a hypertonic solution and in the thawed eggs. The acid formation described above is perhaps identical with the "acid of injury" sometimes referred to.

The inhibition experiments reported above allow certain negative conclusions as to the chemical nature of the acid formed in the broken up eggs. Lactic acid formation is excluded by the iodoacetate experiments, as pointed out already above. The phosphorylation of hexoses is also inhibited by iodoacetate (Lundsgaard, 1930), the dephosphorylation of phosphoglyceric acid by fluoride (Embden and Deuticke, 1935).

The experiments mentioned above on the inhibition of the oxidations of hexosephosphate in the system thawed eggs + pyocyanine by iodo-

[3] In these figures the retention has been accounted for.

acetate and fluoride prove that these compounds interfere with the carbohydrate metabolism of the eggs. Also in dry yeast the aerobic oxidations of hexosephosphate in presence of pyocyanine are strongly inhibited by iodoacetate. Pyocyanine which is without any influence on the acid formation in the broken up eggs favors the formation of phosphoglyceric acid from hexosediphosphate in suspensions of dry yeast according to observations carried out by Lennerstrand and the writer.

The activity of the proteolytic enzyme cathepsin is inhibited by iodoacetate (cf. Bersin, 1935). A proteolytic process induced by this enzyme is excluded as a source of the acid formation in our system.[4] It is to be remembered that iodoacetate does not inhibit the activation process although it could be ascertained that the substance had entered the eggs (Runnström, 1935).

Further evidence of the analogy between the processes induced by freezing and thawing on one side and activation on the other will be presented by Dr. Mirsky. We both find a really significant resemblance between the two processes very probable. The activation "opens doors" (Needham and Needham, 1927) in the interior of the egg. The freezing and thawing breaks down walls allowing mixture of substances separated in the intact unfertilized egg.

I wish to express my sincere thanks to Dr. L. Michaelis for his generous help and valuable criticism and suggestions. I am also very indebted to Dr. A. Mirsky for valuable suggestions and stimulating discussions.

Summary

In previous work it has been found that the activation process is accompanied by an acid formation which has been demonstrated by manometric methods. A comparatively strong acid formation takes place also when the eggs are broken up by distilled water. It seems possible in view of certain observations reported above that the acid formation in both cases has the same chemical basis. The work reported has to be regarded as a first approach to a closer study of this

[4] One objection could be raised against these conclusions. The reaction of iodoacetate or fluoride may be too slow to prevent the acid formation in the thawed eggs. The substances in question were added to the egg suspensions before freezing and the concentrations chosen were considerably higher than those generally used. Iodoacetate in the concentration 0.00054 m inhibits fermentation according to Lundsgaard (1930). Lennerstrand and the writer (unpublished data) found 0.003 m iodoacetate—a concentration ten times lower than that used above—to give an inhibition in suspensions of dry yeast already during the first ten minutes following addition. The inhibition was complete, however, only from the second ten-minute period.

question, which has a bearing on the understanding of the activation process.

The eggs have been broken up in the experiments reported by freezing and thawing. During the thawing a considerable acid formation takes place by far exceeding that observed in the activation process. The acid formation was estimated by manometric determinations of the decrease of combined carbonic acid. In a number of experiments the solid particles of the thawed suspensions were centrifuged down. The fluid part has a lower content of combined carbonic acid than the suspension as a whole. Possibly the proteins of the solid particles combine with carbonic acid under the given conditions. The fluid part of the egg suspensions exercises a visible effect on the jelly surrounding the eggs.

Monoiodoacetate 0.03 m, fluoride 0.06 m, and copper 0.0004 per cent have no inhibiting effect on the acid formation. Addition of hexosemonophosphate does not seem to enhance the acid formation. Iodoacetate in presence of hexosemonophosphate does not inhibit the acid formation. Hence it seems extremely unlikely that the acid formation has anything to do with lactic acid formation. Phosphorylation of hexoses or dephosphorylation of phosphoglyceric acid seem to be excluded by the iodoacetate, and the fluoride experiments. Proteolysis under the action of cathepsin is also excluded by the iodoacetate experiments. An oxidation-reduction system like pyocyanine has no influence on the amount of acid formed.

Suspensions of thawed eggs + hexosemonophosphate + pyocyanine form a system with a fairly high oxygen uptake. This is decreased by 30 per cent on addition of 0.03 m iodoacetate or 0.06 m fluoride. This proves that the two substances in question interfere with the carbohydrate metabolism in this system.

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INFLUENCE OF IODOACETATE ON ACTIVATION AND DEVELOPMENT OF THE EGGS OF ARBACIA PUNCTULATA

JOHN RUNNSTRÖM [1]

(From the Institute of Experimental Zoölogy, Stockholm, Sweden, and the Marine Biological Laboratory, Woods Hole)

Tyler and Schultz (1932) first tried the effect of iodoacetate on fertilization. Their material was the eggs of the worm *Urechis caupo*. They did not find any inhibition of fertilization by 0.02 m solution of sodium iodoacetate.

Runnström (1933*a*) combined 0.002 m sodium iodoacetate with a strong hypertonic solution. The addition of iodoacetate did not change the acid formation following the treatment of the unfertilized *Paracentrotus* egg with the hypertonic solution. Later E. Ellis (1933) tried the influence of iodoacetate on the eggs of *Urechis* and in some few experiments on *Strongylocentrotus purpuratus*. Ellis claims that the iodoacetate is without any influence on the fertilization and division of the egg. The concentrations of the iodoacetate used varied from 0.001–0.01 m. In the fertilization experiments the eggs were placed for five minutes in the solution and then inseminated. In these experiments, as in those by Tyler and Schultz, the question is whether the iodoacetate had time to penetrate the unfertilized eggs before insemination. Ellis proved that iodoacetate penetrates the *fertilized* eggs after an exposure for 95 minutes to an 0.01 m solution of iodoacetate. The glutathione content is estimated in the exposed eggs and found to be only half of the quantity found in the control. One can not take for granted from these figures that the iodoacetate penetrates the unfertilized eggs in five minutes.

R. S. Lillie (1931) mentions that iodoacetate in the concentration of 0.02 per cent (about 0.001 m) is without influence on the heat activation of starfish eggs. Lillie considers the possibility that the iodoacetate does not penetrate under these conditions.

In the present experiments a comparatively high concentration of the iodoacetate (0.03 m) was generally used. The eggs were treated for several (2–6) hours with the iodoacetate containing sea water: during the treatment the eggs were gently shaken to facilitate the diffusion

[1] Fellow of the Rockefeller Foundation, 1933–1934.

processes. In spite of this the eggs could be fertilized in the iodoacetate sea water. This happened even if the pH of iodoacetate + sea water had been lowered considerably (from 8.1 to 6). In this medium the iodoacetate will probably penetrate more rapidly because of the formation of undissociated molecules of iodoacetic acid. The cytolysis is indeed very strong after two hours treatment but the few eggs still remaining intact can be fertilized after transfer to normal sea water. A normal membrane formation ensues. The microscopic examination revealed in many cases that the eggs although still fertilizable were beginning to present marked structural changes such as precede cytolysis. Eggs in sea water at pH 6 without iodoacetate do not cytolyze. Eggs exposed to iodoacetate for 2–6 hours were subsequently transferred to normal sea water and fertilized. The larvæ developing from these eggs have *shown definite morphological defects*. This proves that the iodoacetate penetrates the unfertilized egg.

The evidence submitted above shows that none of the enzymatic processes inhibited by iodoacetate are necessary for the activation of the egg. Among these glycolysis is especially important; also the proteolytic enzyme cathepsin is inhibited by iodoacetate, as well as all enzymatic or other activity in which the presence of free SH-groups is necessary (cf. the discussion in the recent papers by Michaelis and Schubert, 1934, and Hellerman and Perkins, 1934).

It may be mentioned that the sperm-agglutinating substance secreted by the egg is unaffected by iodoacetate. A series of dilutions was made according to the method of Frank Lillie (1914). No difference in the behaviour of the sperm towards the egg water in presence and absence of iodoacetate could be observed. SH-groups are therefore not involved in the activity of the sperm-agglutinating substance.

The eggs inseminated in iodoacetate show a normal or almost normal increase of respiration upon fertilization. But soon a decrease of the respiration ensues. The presence of 0.002 m sodium pyruvate removes almost completely the inhibition of the respiration by iodoacetate in fertilized eggs. In one experiment, July 23, 1934, the oxygen consumption was measured by the Warburg manometric method, using (1) a control, (2) a sample with addition of 0.03 m iodoacetate, and finally (3) a sample with addition of 0.03 m iodoacetate and 0.02 m sodium pyruvate. The suspension was 6.5 volumes per cent. Each vessel contained 3 cc. egg suspension.[2] The oxygen consumption was, in the three vessels 1, 2, 3, respectively: 133, 88, 122 cu. mm. The average residual respiration in the iodoacetate was 66 per cent. During

[2] The measurements were begun at the time of fertilization of the eggs and continued for 100 minutes.

the first 30 minutes following fertilization the residual respiration was 81 per cent and during the last 30 minutes of the measurement the residual respiration had decreased to 45 per cent. In the mixture iodoacetate + pyruvate the decrease is very small and is due indeed to an inhibition, which was appreciable only during the period 70–100 minutes after fertilization. The development is also blocked by 0.03 m iodoacetate. In the quoted experiment a retardation of the development in presence of iodoacetate was very pronounced. Segmentation took place, but the cells died after some hours even if transferred to normal sea water. The hyaline plasma layer is affected by the iodoacetate. The cells are detached from each other. In some experiments the development was arrested by iodoacetate in a still earlier stage. In one experiment, July 20, 1934, 95 per cent of the eggs remained in the early prophase of the first division as after exposure to n/5,000–n/10,000 HCN. On the living material this stage can be identified by the formation of the "clear streak" close to the nucleus, the contour of which is clearly visible (cf. Fry and Parks, 1934, Fig. 6). In the experiment of July 20, 1934, the inhibition of the development by iodoacetate was thus stronger than in the experiment of July 23, 1934, referred to above. It may not be a mere coincidence that the respiration is also more strongly inhibited in the experiment of July 20, 1934. The oxygen consumption was, during 80 minutes, 124 cu. mm. in the control, and only 50 cu. mm. in presence of 0.03 m iodoacetate. The average residual respiration is 40 per cent. During the first 10 minutes following fertilization the residual respiration is more than 80 per cent, i.e. the initial increase of the respiration is almost normal. In this experiment 0.02 m sodium lactate was added, and again the respiration was restored to a value not far below that of the control 114 cu. mm., i.e. residual respiration 92 per cent. The suspension in this experiment was 7.2 volumes per cent. The number of eggs arrested on the stage of the first prophase in the mixture of sea water, iodoacetate, and lactate was 87 per cent, a very slight improvement of the development indeed (cf. above the value for the iodoacetate without lactate). In no case did a development to swimming larvæ take place in the 0.03 m iodoacetate solutions in sea water.

The experiments reported show that iodoacetate affects the carbohydrate metabolism in the sea urchin egg in the same way as in other cells. A part of the respiration may be due to an oxidation of carbohydrate breakdown products. When the formation of these is inhibited the respiration may decrease. But it is evident from the experiments with fertilized eggs that iodoacetate interferes also with processes in the cell other than glycolysis as a normal development is not established by

the addition of pyruvate or lactate. The addition of lactate or pyruvate, although restoring the respiration, does not save the eggs from the injurious effect of iodoacetate.

Fertilized eggs are decidedly more sensitive to iodoacetate than unfertilized eggs. This may be due to a considerable increase of permeability of the egg to iodoacetate upon fertilization, or it may be due to an increased reactivity of the egg cytoplasm with iodoacetate upon fertilization, or to a combination of these two changes.

As mentioned above, the larvæ developing from eggs treated with 0.03 m iodoacetate *before* fertilization present definite morphological defects. The stomodæum and the oral arms are poorly developed, sometimes lacking, while the organisation and size of the pluteus is in all other respects normal. Addition of 0.02 m sodium pyruvate to the sea water in which the larvæ develop removes almost completely the effect of the treatment by iodoacetate applied before fertilization. Hence it seems probable that a carbohydrate breakdown is a necessary condition in the morphological differentiation of the anterior or "animal" part of the larva. This conclusion is in good agreement with the results obtained by Lindahl (1934, 1935). This author thinks it probable that lithium interferes with some part of the carbohydrate metabolism of the egg. As is well known, lithium salts added to the sea water even in fairly low concentration (e.g. 0.1 per cent LiCl) has a strongly inhibitory influence on the morphological differentiation of the animal region of the larva. A certain decrease of respiration by exposure of the developing eggs to a mixture of 95 per cent carbon monoxide and 5 per cent oxygen also impairs the morphological differentiation of the animal part (Runnström, 1928, 1933*b*). Thus the carbohydrate metabolism must take place under strictly aerobic conditions to warrant a normal morphological development of the animal region of the larva.

This work was carried out during the summer of 1934 at the Marine Biological Laboratory, Woods Hole. My thanks are due to Dr. L. Michaelis for his generous help and valuable suggestions.

Summary

1. It has been proved by the experiments briefly reported above that monoiodoacetate does not inhibit the fertilization process in the *Arbacia* egg. Thus enzymatic or other activities in which SH-groups are involved do not play an essential rôle in the fertilization process. The activity of the sperm-agglutinating substance secreted by the egg is not impaired by iodoacetate. The penetration of iodoacetate into the unfertilized egg is ascertained by the presence of morphological defects in

larvæ reared from eggs removed from the iodoacetate sea water and fertilized in normal medium.

2. Iodoacetate in the concentration (0.03 m) used in these experiments is harmful to the development of fertilized eggs. There is a remarkable difference in resistance between unfertilized and fertilized eggs towards iodoacetate. The normal increase of respiration following fertilization takes place also in 0.03 m iodoacetate but there is soon a considerable decline of respiration, which can be prevented by addition of pyruvate or lactate. A carbohydrate metabolism must be involved in the oxidation processes in the fertilized eggs. The addition of pyruvate or lactate, although restoring respiration, does not inhibit the injury caused to the fertilized egg by the addition of iodoacetate.

3. The morphological defects caused by a treatment of the eggs by iodoacetate before fertilization are prevented by addition of pyruvate to the fertilized eggs. These results have some bearing on the development mechanics of the sea urchin. A carbohydrate breakdown seems to be a necessary condition for the full morphological differentiation of the anterior or animal region of the larva.

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DIFFERENTIAL OXYGEN UPTAKE OF REGIONS OF LIMULUS OPTIC NERVE AS RELATED TO DISTANCE FROM THE SENSE ORGAN

RITA GUTTMAN

(From the Marine Biological Laboratory, Woods Hole, Mass., and the Zoölogical Laboratory, Columbia University)

Differences in manifestation of physiological activity along the length of a nerve have been the subject of considerable study. In 1933, Marshall and Gerard found that in the most rapidly conducting fibres of the bullfrog sciatic nerve, the velocity of conduction is 27 per cent less in the distal fourth than in the proximal fourth. They found that in the green frog sciatic nerve the rate increases slightly from the proximal to the middle portion and then decreases peripherally. It was thought that a study of respiratory rate along the length of a nerve might also be of interest. The problem is relevant when considered in relation to the question of the nature of the nerve impulse. If the nature of the nerve impulse is not purely physical and is a wave of chemical change, then it is conceivable that metabolic activity may influence the velocity with which the impulse sweeps down a fibre.

The nerve selected for study was the *Limulus* optic nerve rather than the frog sciatic, since for reasons to be elucidated later the former is somewhat better material for differential respiration studies.

In a study of the carbon-dioxide production of the lateral line nerve of the dogfish, Parker in 1928 concluded that no gradient existed in that nerve since the two halves differed but slightly and then not consistently in regard to direction of difference.

Gerard in 1927 investigated the frog sciatic nerve and could demonstrate no respiratory gradient there. On the other hand, Tashiro and Adams (1914), working with an optical method of determination of barium carbonate precipitation for measurement of carbon-dioxide production, claimed that a respiratory gradient existed in the optic nerve of the king-crab, *Limulus polyphemus*. Child (1921) cites the work of Tashiro and adds that differences in rate of metabolism in nerves play a very important part in determining electrical gradients.

Inasmuch as the results of Tashiro and Adams on carbon-dioxide production are high compared with later work and inasmuch as they divided the nerve into but two parts in studying the possibility of the

existence of a gradient, it was decided to investigate in detail the oxygen uptake of regions of *Limulus* optic nerve with the Gerard-Hartline microrespirometer (1934) (an apparatus with which the respiration of as small an amount of tissue as 1 mg. may be observed directly from minute to minute) and to divide the nerve into five parts for study instead of only two. The present paper is a report on thirteen experiments.

The optic nerve of *Limulus polyphemus* is one of the longest and most uniform in diameter among the invertebrates, and its fibres are non-medullated. It varies between about eight and twelve centimeters in length, and is protected along its entire course by a uniform non-nervous sheath, which was not stripped from the nerve in these experiments. Male *Limuli* were used since the dissection is easier there. The nerve was obtained intact from the sense organ to the brain in most cases. Dissection was always begun at the eye and proceeded centrally. Every effort was made to avoid undue manipulation.

The nerve was cut into five approximately equal segments and each piece inserted by means of a fine hooked glass needle into a calibrated glass capillary. One end of the capillary was sealed with vaseline and at the other end a drop of half molar sodium hydroxide was inserted. The capillary was then placed in a sealed glass chamber of much larger volume and the whole immersed in a constant temperature water bath. Movement of the drop was observed with the aid of a horizontal microscope equipped with a filar-ocular micrometer.

Carbon-dioxide evolved was absorbed by the alkali, which also acted as an index drop, and since the ratio of the volume of the outer glass tube to the capillary volume was more than 5000 to 1, the rate of movement of the drop gave a direct measurement of the oxygen uptake.

Instead of studying the respiration of cut sections of the nerve, it would have been more desirable to study the several regions in the intact excised nerve, or better still, *in situ* in the living animal. Since the technique for this has not been perfected, the next best procedure was adopted.

The typical respiration curve of a nerve or portion of a nerve is plotted in Fig. 1. Owing to the recent handling and dissection, respiration is high at first, then drops to a uniform steady rate which is maintained for about five or six hours, falls again when the oxygen present is insufficient to support maximal respiration, and then vanishes. The respiration studied in these experiments is the uniform steady rate.

In discussing his results with Dr. Tashiro, I discovered that the aspect of gas exchange he was studying was the high activity represented in the first part of the curve, since he measured carbon-dioxide production

immediately after dissecting out the nerve and for a short period of time —usually not more than fifteen minutes. This may explain the differences in his results and the results obtained in these experiments as regards both relative and absolute rates. In regard to relative rates, different amounts of time are required to achieve a steady state in different nerves and regions of nerves. With respect to absolute rates, results would obviously be higher in his experiments if the first part of the curve only were utilized.

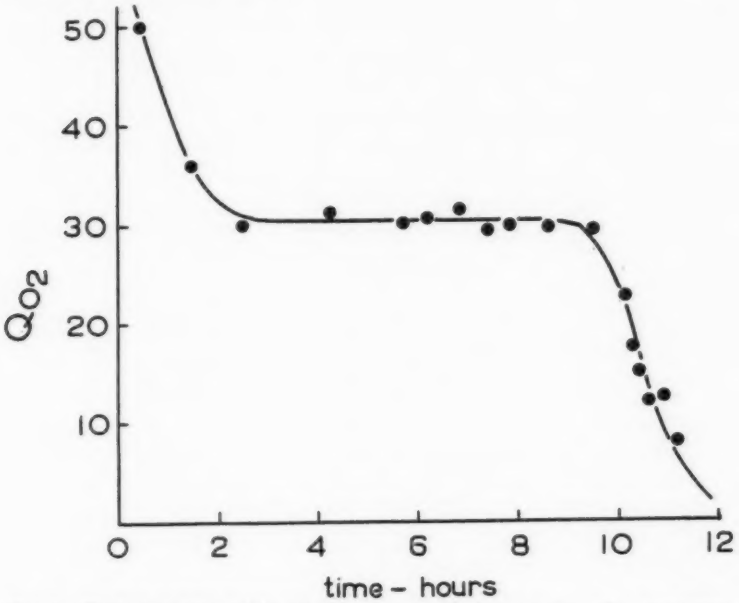

FIG. 1. Typical oxygen uptake of a nerve or portion of a nerve. Q_{O_2} represents cu. mm. oxygen per gram moist weight per hour.

That the high portion is not an artefact entirely due to movement of the drop in reaching an equilibrium was shown by control experiments where sodium hydroxide was inserted into a capillary containing no tissue.

The steady rates of the five portions of a nerve were compared. The results are summarized in Table I. "*A*" represents the fifth of the nerve most proximal to the brain; "*E*", the fifth of the nerve most distal to the brain, and "*B*", "*C*" and "*D*", intermediate regions. In every case where the most proximal fifth is present (in two early cases, Experiments 11 and 13, the nerve was not dissected out entirely to the

brain) the proximal fifth has a higher respiratory rate than the distal one. In every case except the last two, maximal respiration occurs between the two ends of the nerve. The average difference between maximum and minimum is 36 per cent, which is far beyond the precision of the instrument.

Both right and left nerves were studied. In Experiments 1, 2 and 3, 4, which represent paired nerves, no striking differences were observed between right and left nerves. Respiration of the forebrain was studied in two cases and found to be higher than in any part of the nerve.

Tashiro and Adams (loc. cit.) based their belief in the existence of a gradient in the optic nerve of *Limulus* on observations that the respiration of the distal half was higher than the respiration of the proximal half. Yet if we compare the distal two-fifths with the proximal two-fifths in these measurements, we find the reverse situation to be true, i.e. not the distal but the proximal two-fifths is found to have the higher respiratory rate in each case. It was partly on the assumption that respiration of a nerve is highest at the point of stimulation, i.e. the sense organ, that Tashiro based his theory of nerve conduction outlined in "A Chemical Sign of Life" (1917).

The effect of temperature upon the differential respiratory rate was also investigated. The relationships found above remained unaltered throughout the temperature range: 16 to 31° C.

Q_{O_2} of regions of Limulus optic nerve

Expt.	Temp. (°C.)	Brain → Eye					Prox. 2/5	Dist. 2/5
		A	B	C	D	E		
1	31.0	99.7	109	118	95.0	82.5	105	89.0
2	31.0	102	92.0	117	101	81.0	98	91
3	28.3	65.1	62.5	76.1	53.4	53.3	63.7	53.3
4	28.3	68.1	67.6	76.1	55.6	47.3	67.9	51.5
5	19.3	36.4	49.9	40.6	43.4	34.6	43.1	39.0
6	18.0	28.5	32.9	34.4	25.1	23.7	30.8	24.5
7	17.1	30.0	38.6	30.6	27.8	25.2	34.4	26.6
8	17.1	40.1	36.3	?	29.8	32.4	38.2	31.1
9	16.1	15.3		17.7	16.5	9.4	15.3	13.0
10	16.1	32.2	32.3	23.2	27.8	15.2	32.3	21.6
11	16.1	—	14.7	19.8	19.0	12.9	—	—
12	16.1	32.4	30.7	29.5	31.1	20.9	31.5	26.0
13	16.1	—	23.6	18.1	21.2	31.6	—	—

A is most proximal fifth. *E* is most distal fifth.

Q_{O_2} represents cu. mm. oxygen at 0° C. per gram of tissue (moist weight) per hour.

Summary

The differential rate of oxygen uptake of the optic nerve of *Limulus polyphemus* was investigated by means of a differential volumeter. Thirteen experiments are reported. It was found that:

1. In every case the respiration of the distal end was lower than that of the proximal region. The respiration of the distal end was lower than that of any other region of the nerve in all the cases studied but one.

2. A maximal rate was found in ten out of twelve cases along the central portion of the nerve. Thus no gradient in the accepted sense of the word of oxygen uptake exists in the nerve.

3. The average difference between maximal and minimal rates in a nerve was 36 per cent.

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CHANGE IN RATE OF RESPIRATORY METABOLISM IN A TELEOST FISH INDUCED BY ACCLIMATIZATION TO HIGH AND LOW TEMPERATURE

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Introduction

The possibility that the rate of oxygen metabolism of fishes at any given temperature may be conditioned by the temperature of the water from which the animals are taken seems not to have been considered in the work so far published on oxygen metabolism of fishes. The probability that a change in the rate of metabolism of cold-blooded vertebrates would be effected by acclimatization of the animals to different temperatures has been expressed by Montuori (1913), Krogh (1916), Baudin (1932), and Benedict (1932). Krogh reasons that since fishes are found living normally over such a wide temperature range, there must be some adjustment of the metabolic processes to temperature, that "animals living at a very low temperature should show a relatively high standard metabolism at that temperature compared with others living normally at a high temperature."

Benedict finds from his study on the metabolism of reptiles that the warmer the environment from which the animals are procured, the lower is their standard metabolism. He states, however, that "the effect of geographical location of the animal cannot be considered to be clearly demonstrated, since the size of the various species is shown to play a considerable rôle."

In another publication (Wells, 1935*b*) I have discussed the change in the metabolic rate of *Fundulus parvipinnis* after these fishes had been subjected to high and low temperatures for definite intervals of time. This paper is based upon a continuation of that work, using another species of fish and employing greater temperature differences between the cold and warm-adapted fishes.

Material and Methods

The fishes used in these experiments were *Gillichthys mirabilis*, known to fishermen as "mud suckers," and used extensively as bait because of their extreme hardiness. They will live equally well in fresh or salt water and will remain alive for hours out of water if they are

kept in a moist environment. Ordinarily they may be kept indefinitely under laboratory conditions since they are not subject to the disease which is so fatal to *Fundulus* (Wells and ZoBell, 1934). They remain healthy and normal over a wide temperature range (from 8° to 35°).[1]

The water for the warm aquaria was heated by passing it through a block tin coil sealed into an ordinary hot water tank. It was found necessary to remove the excess of dissolved gases from the sea water after it had been heated, because at the high water temperatures the supersaturated sea water caused the well-known "gas bubble disease" of fishes. The excess gases were removed by passing the water in a very thin layer over a cascade constructed from two ordinary glass washboards. Since the water temperature was approximately three degrees higher at the top of the cascade than it was when it reached the tank, the method was quite effective and resulted in maintaining the water in the stock tanks slightly below the saturation point at any desired temperature.

The cold water was obtained by passing the sea water through a block tin coil immersed in a brine tank, cooled with an electric refrigeration unit. With heating and cooling devices it was possible to maintain running sea water at temperatures from 8° to 40°.

Elsewhere (Wells, 1935*a*) the apparatus used for the determination of the oxygen metabolism of fishes has been described in detail. Briefly, it provides a continuous flow, constant temperature system, in which the rate of flow can be maintained at any desired value, and the temperature can be controlled to within 0.1° C. Oxygen was determined by the well-known Winkler method.

Experimental Procedure

In December, 1934, two groups of 9 fishes each,[2] of as nearly equal mean size as it was possible to select, were placed in respiratory chambers. The first group, to be designated A_1, was taken from a tank in which the running sea water had been maintained between 32.8° and 34.5°, but usually at 33°, for a period of 17 days. Group A_2 had been kept for the same interval of time in cold water the temperature of which varied from 10.5° to 13°, but during most of the time the temperature was *ca.* 11°. Both groups were placed in respiratory chambers (see Wells, 1935*a*, p. 205) and the chambers immersed in the constant temperature bath at a temperature of 22°. The first value of their oxygen consumption was taken 30 minutes later. Other values were obtained during the daytime for a period of 50 hours. These values are plotted in Fig. 1, *A*.

[1] All temperatures are expressed in ° C.

[2] The average weight of the *A* group was 8 grams and of the *B* group, 8.9 grams.

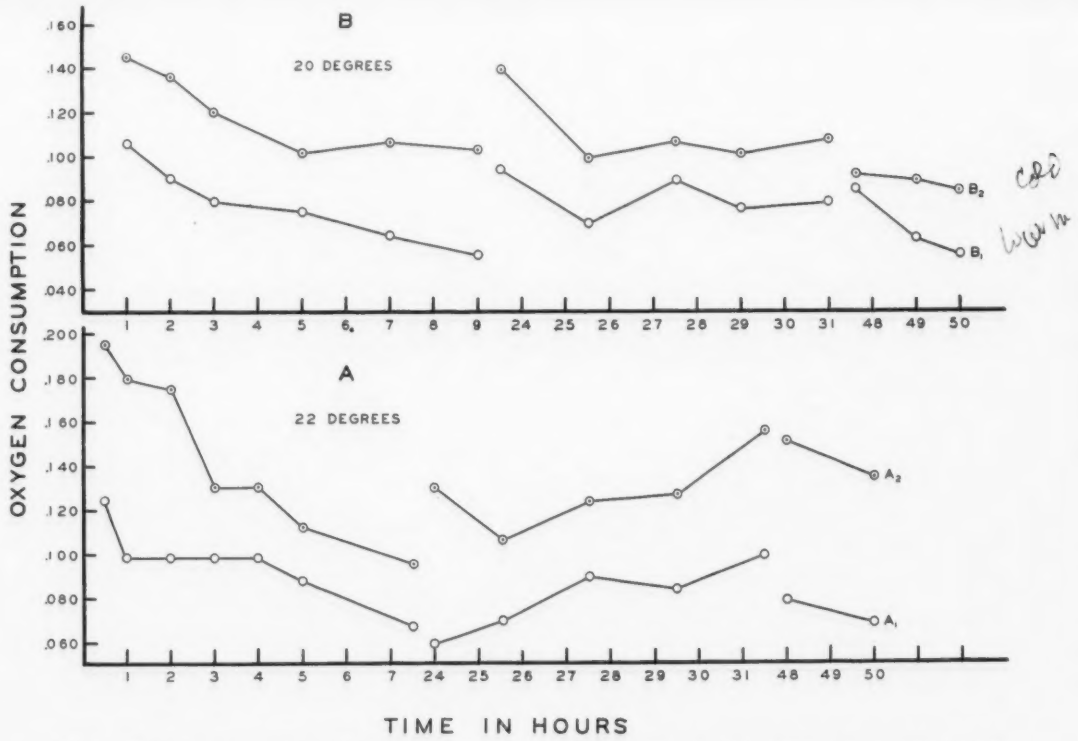

FIG. 1. Oxygen consumption at a common temperature of fishes acclimatized to different temperatures. The ordinates represent the oxygen uptake of the fishes in grams per hour at a temperature of 22° in *A* and 20° in *B*. The abscissas represent time in hours from the moment of transfer of fishes to the respiratory chambers. The upper curves in both *A* and *B* are the values obtained from the fishes of cold water history, and the lower curves, the values from the ones of warm water history.

On January 3, two more sets of fishes (Experiment 2), 16 in each set, were selected. The warm-adapted fishes, group B_1, had been kept in running sea water the temperature of which varied from 29.6 to 31.6 for a period of 20 days, and group B_2, the cold-adapted fishes, had been maintained at a temperature level between 10° and 12° for the same interval of time. In this experiment, since the temperature of the acclimatization period of the "warm" fishes had been 2½ degrees lower, the metabolism values were obtained at 20°. The first value was secured one hour after introduction into the respiratory chambers and, as in the first experiment, values of the oxygen consumption were obtained throughout the daytime for 50 hours. These values are plotted in Fig. 1, *B*.

In both of these experiments a standard feeding schedule was adopted. The fishes in each group were fed all the live food they would take during the period of acclimatization and were deprived of food for an interval of 24 hours prior to being placed in the respiratory chambers.

Discussion and Results

There is undoubtedly a change in the "normal metabolism" (Wells, 1932) of fishes effected by acclimatizing them to high and low temperatures. An excellent visual indication of this is observed when comparing the opercular breathing rhythm of two groups of fishes from cold and warm water at a common intermediate temperature. Fishes with a cold water history, even after 10 days at the intermediate water temperature, breathe at nearly twice the rate of that of the warm history fishes. A detailed discussion of the change in opercular breathing rhythm and the resistance to anæsthetics, asphyxiation, and cyanide poisoning produced by acclimatizing fishes to high and low temperature, will be found elsewhere in this journal (Sumner and Wells, 1935).

In another paper (Wells, 1935*b*), I have presented the data to show that there is not only a change in the rate of respiratory metabolism of *Fundulus parvipinnis* when these animals are acclimatized to high and low temperatures, but that there is a seasonal rhythm even when these fishes are maintained at a constant temperature. The seasonal change, in view of the results presented in this paper, leads one to the conclusion that the fish possesses a buffering mechanism, so to speak, which enables it to adjust itself to the seasonal changes in water temperature, thereby maintaining a practically constant rate of oxygen metabolism. My reason for this statement is, that as the temperature difference to which the fishes are acclimatized increases, the oxygen consumption, at a common temperature, of the cold-adapted fishes increases over that of the "warm" fishes. In my experiments on *Fundulus,* where I was only

able to maintain a temperature difference of approximately 15°, the metabolism of the cold fishes was 20.4 per cent higher at a temperature of 12° than the warm fishes. In the experiments presented in this paper, however, where the acclimatization temperatures were farther apart, the difference between the two groups at a common temperature is greater. In the first experiment (Fig. 1, *A*) the acclimatization temperature difference was, on an average, 22° and the average increase of the cold fishes over the warm ones, after 24 hours in the respiratory chamber, was 67 per cent at 22°. In the second experiment (Fig. 1, *B*), when the acclimatization temperature difference was approximately 19.5°, the increase in oxygen consumption of the cold fishes at a common temperature of 20° was only 37 per cent.

Now let us consider the data presented in Fig. 1. The ordinates represent the oxygen uptake of the fishes in grams per hour at a temperature of 22° in *A* and 20° in *B*. The abscissas represent time in hours from the moment of transfer of the fishes to the respiratory chambers. The upper curves in both *A* and *B* are the values obtained from the fishes of cold water history and the lower curves are values obtained from the ones of warm water history. It is obvious from an inspection of these two sets of curves (Fig. 1) that the oxygen metabolism of the warm-adapted fishes is lower than that of the cold-adapted ones when determined at the intermediate common temperatures. In every case the simultaneously determined values of the warm-adapted fishes fall below those of the "cold" fishes. With few exceptions, the trend of each curve in a pair is the same.

In the *B* series curves, where the values were obtained at 20° and the warm fishes had been maintained at a 2.5° lower acclimatization temperature than in the *A* series, there is an indication that the induced difference of metabolic rate of the two temperature groups is disappearing. In the *A* groups, on the other hand, the values on the second day are farther apart than on the first, and still farther on the third day. The increase on the third day of the cold fishes over the warm ones was 98 per cent. This difference was substantiated in the differential reaction of these two groups to urethane after the fishes were removed from the respiratory chambers (Sumner and Wells, 1935). It must not be overlooked that these data are too meager to justify concluding that the 2.5° acclimatization temperature difference is entirely responsible for the difference in reaction shown in these two experiments. The difference between the reaction of the two sets, *A* and *B*, may be due, in part, to the different temperatures at which the values of the respiratory metabolism were determined. I have shown elsewhere (Wells, 1935*a*) that the relation of oxygen consumption to temperature, in fishes, does not fol-

low a smooth curve, but may increase markedly at certain temperatures; and, further, that the temperatures at which these increases occur may be conditioned by the temperature of the water from which the fishes are taken, that is to say, the temperature to which the animals have been acclimatized. It is obvious from a comparison of the two graphs *A* and *B* that the rate of oxygen consumption was less stable or constant at 22° than it was at 20°. This is indicated by the greater variability of the consecutive values of both the cold and warm water fishes.

However, a point that seems to me to have significance is the fact that the warm-adapted fishes, acclimatized to a 2.5° higher temperature in the *A* series than in the *B* series, had practically the same rate of metabolism at 22° as the *B* series had at 20° (.079 and .077 respectively) [8] while the cold-adapted fishes in the *A* series were 28 per cent higher at 22° than the cold-adapted *B* series at 20°. If, as I have postulated, these fishes are able to adjust their rate of metabolism to temperature changes, these results are predictable. The *A* and *B* series warm fishes have been subjected in both cases to an effective drop in temperature of approximately 11°; therefore, if their rates of metabolism at the two high temperatures had been established at essentially the same level, the 11° drop should affect the two groups of fishes equally. Both sets of cold fishes, on the other hand, had been acclimatized to the same temperature, and the effective increase in temperature was two degrees higher for the *A* series than for the *B* series, and the metabolism of the *A* group at 22° should be higher than the metabolism of the *B* group at 20°.

The data presented in this paper certainly warrant considering that at least two species of fishes are able to achieve a certain amount of adjustment of their oxygen metabolism to the temperature changes of their environment. How rapidly and to what extent they can make the adjustment is yet to be determined. We have an indication from the breathing rhythm (Sumner and Wells, 1935) that an adjustment once made persists for several days. The permanency of the adjustment is no doubt a function of the duration of time at which the animals are maintained at the high or low temperatures. These results, in conjunction with the results of Sumner and Wells, on the differential effect of anæsthetics, asphyxiation, and cyanide poisoning on fishes acclimatized to different temperatures, indicate that further work would be highly desirable.

[8] The rates of metabolism are obtained by averaging all values secured after the fishes had been in the respiratory chambers for a period of 24 hours (see Wells, 1932).

SUMMARY

The results of two experiments are presented showing the effect on the oxygen metabolism produced by acclimatizing fishes to high and low temperatures. It is concluded from an analysis of these data that fishes acclimatized to a high temperature have a much lower rate of metabolism at a common intermediate temperature than do fishes acclimatized to a low temperature, and that the magnitude of this difference is a function of the difference between the acclimatization temperatures.

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SOME RELATIONS BETWEEN RESPIRATORY METABOLISM IN FISHES AND SUSCEPTIBILITY TO CERTAIN ANESTHETICS AND LETHAL AGENTS

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Introduction

It has long been known that gradual acclimatization may enable an animal to withstand temperatures so high that they would speedily prove fatal were the change made abruptly. There is some evidence, though of less extent, relating to extreme reductions of temperature. It is a curious fact that tolerance to high temperatures, once acquired, may persist for considerable periods after the return of the organism to the temperature from which it was taken, or even to a considerably lower one. Some rather lasting, though probably not permanent, change has been brought about in the physical or chemical properties of the organism. Regarding the nature of this change, various hypotheses have been offered. Certain authors have suggested the probability of a rise in the coagulation point of proteins, due to a loss of water in the tissues, as a result of exposure to higher temperatures.[1] Others have pointed to the rôle played by differences of metabolic rate in determining the resistance of organisms not only to heat but to other lethal agents.

Experiments by Child (1913 and later) "demonstrate the existence of a relation between the length of life (physiological resistance) of the animals or pieces in KCN, alcohol, and various other anesthetics and the rate of the metabolic reactions or certain of them, probably the oxidations." Miss Behre (1918), working in Child's laboratory, found for a planarian that transfer to a higher temperature increased the metabolic rate and, conversely, that transfer to a lower temperature decreased this. Much more important, for present purposes, was the fact that any such transfer was followed by a regulative process, so that a worm which had been kept for a certain length of time at a given temperature displayed a definitely higher rate of metabolism than one which was transferred to this directly from a higher temperature, and a definitely lower rate than one which was transferred directly from a lower temperature. The indices of metabolic rate employed by her were susceptibility to poisoning by KCN, and the production of CO_2.

[1] Davenport and Castle, 1895.

Loeb and Wasteneys (1912), while not concerning themselves with respiratory metabolism, conducted some highly instructive experiments with *Fundulus* (presumably *F. heteroclitus*), a small marine teleost. Their objects were, among other things, "to find out the maximum temperature into which they [fishes kept at 10° to 14° C.] could with impunity be [transferred] suddenly" and "how long it takes to immunize the fish against the harmful effects of a sudden transfer to a temperature of 35° C."

Probably the most striking results from these experiments relate to the high persistence of the immunity obtained, in comparison with the duration of the immunization process. Thus, "the immunity against a temperature of 35° acquired by keeping the fish for two days at 27° is not lost or weakened if the fish are afterwards kept as long as thirty-three days at a temperature of from 10° to 14°." (Experiments not continued longer than this.) More surprising yet, perhaps, "the immunity against a temperature of 35° is also maintained if the fish are kept after the two days' exposure to 27° for two weeks at a temperature of 0.4° C." Moreover, "it is not necessary to expose [fishes] continuously to a high temperature. An intermittent exposure to a higher temperature during a number of hours each day will bring about the same effect." (See, also, Hathaway, 1928.)

Wells (1935, 1935a, 1935b) has measured the consumption of oxygen for another species of *Fundulus* (*F. parvipinnis*) and for one species of goby, under various conditions of temperature and season. He has shown not only that the "metabolic rate," as measured by oxygen consumption, is a function of the temperature at the time of the experiment, but that the rate for any given lot of fishes is greatly influenced by the recent temperature history of the animals in question. Thus, in one experiment with gobies, two lots of nine fishes each, which had been kept for 17 days at approximately 11° and 33.5° C., respectively, were tested after both lots had been held at a common, intermediate temperature for 48 hours. The former lot gave a figure for oxygen consumption almost exactly twice as great as the latter (Wells, 1935b).

The agreement between these results with fishes and those obtained by Miss Behre with planarians is obvious. The suggestion is also obvious that the physiological differences which express themselves as differences in oxygen consumption may be directly related to, if not identical with, those upon which differences in immunity to high temperatures depend. If a fish which has been freshly subjected to a much higher temperature exhibits, at this temperature, a higher rate of respiratory metabolism than a fish which has been acclimatized to this condition, it is not surprising that the former fish should be more likely to

succumb than the latter. For it does not seem improbable that one factor, at least, in the lethal effect of high temperatures is a fatal increase in the rate of oxidation.

Loeb and others have attributed to cyanides an effect in reducing the ability of living matter to utilize oxygen, while Child has employed the relative susceptibility of an organism, or part, to potassium cyanide and some other substances as a useful index of its metabolic rate. It therefore seemed to us desirable that fishes having different histories in relation to temperature should be subjected to the influence of certain lethal agents, in order that comparisons might be made among these fishes in respect to their susceptibility.

Although about a hundred experiments of this sort have already been made by us, the present report is to be regarded as a "preliminary" one, even though it may not be possible to continue these studies in the future. Many essential phases of the subject have been left untouched, and certain lines of experimentation have not been brought to a conclusive issue. We have been handicapped, at times, by lack of material, and the control of water temperature was not always adequate. However, no reservations seem necessary as regards our major lines of evidence. In general, we may say that the "horizontal" comparisons, i.e., comparisons of the performance of contrasted lots of fishes at any one time, are of much more importance than "vertical" comparisons, made between corresponding lots of fishes in different experiments conducted at different times. This, because of probable seasonal changes in the fishes; likewise, because of temperature changes in both our "warm" and "cold" tanks, and particularly in the "intermediate" tanks which commonly held untreated sea water.

The temperature of the "cold" aquaria varied somewhat with the season, but lay, nearly always, between 10.5° and 12.0° C., rising occasionally above 13°. The temperature of the "warm" aquaria, in most experiments, lay between 32° and 35°, though it surpassed both of these limits on a few occasions. The duration of subjection to these temperature extremes varied from 24 hours to several months. Except for a brief period, the "intermediate" tanks were supplied with sea water directly drawn from the laboratory supply. This naturally varied with the season, ranging from 15° to 20.5° [2] during the period covered by these experiments.

The fishes used in these experiments were our local killifish, *Fundulus parvipinnis*, and the long-jawed goby, *Gillichthys mirabilis*. Those of both species varied considerably in size, the former ranging from 6

[2] 20°–23° during a few weeks in winter, when it was artificially warmed.

to 8 centimeters in length, the latter from 8 to 15 centimeters. So far as possible, comparisons were restricted to fishes of approximately the same size.

In the greater number of our experiments, our procedure consisted in keeping fishes for periods of varying length in the three tanks just referred to, then returning them to a common temperature and subjecting them to the various anesthetics or lethal agents. In a limited number of the experiments the fishes were tested at the temperatures to which they had become acclimatized. As anesthetics, ethyl urethane (commonly a 1 per cent solution,[3] chloretone (1/20 to 1/10 of saturation), and ether were used; as lethal agents NaCN (1/1,000 M), KCN (1/3,000 M), de-oxygenated sea water, and in one experiment, heat. The time when each fish succumbed to the treatment in question was recorded. A fish was regarded as having "succumbed" when every visible trace of activity had ceased. Since the respiratory movements of the mouth and opercula persisted after other movements had ceased, these were carefully watched. In experiments with cyanide and with de-oxygenated sea water, responsiveness to poking with a glass rod was taken into consideration. With each agent employed, the moment of "succumbing" was commonly fairly well defined, and no serious difficulty was encountered in determining it. "Succumbing," in this sense, was by no means equivalent to death, however, since many of the fishes recovered completely when returned to fresh sea water. Indeed, one of the important data which we recorded in many of our experiments was the recovery or non-recovery of each individual fish when removed from the adverse conditions.

Another important item of observation was the rate (number per minute) of externally visible respiratory movements made by the mouth and opercula. Strongly correlated with the rate of these movements was their depth, differences in which could be observed in most cases, though they could not be quantitatively recorded.

Experimental Results

In the present circumstances, no full record of the various experiments seems called for. The results may be summarized rather briefly. It is hoped that a more detailed report, based upon a more adequate series of experiments, may be presented in the future.

The first experiments to be mentioned are two which related to the susceptibility of fishes to the agents in question, while kept at the temperatures to which they had become acclimatized. In tests with a few

[3] This was prepared by mixing one part of a 10 per cent solution of urethane in distilled water with 9 parts of sea water.

specimens each of *Fundulus* and *Gillichthys*, the fishes in the "warm" tank proved to be consistently more susceptible to urethane than those in the "cold" tank. Rather unexpectedly, however, the fishes in the "intermediate" tank showed a higher resistance than either of the other lots, but it must be stated that the temperature of the "intermediate" water was at this time much closer to that of the "cold" than to that of the "warm."

These experiments are unfortunately altogether too limited in number. It should be pointed out, however, that the results are completely in accord with the observed respiratory rates of the "warm" and "cold" fishes in their own media. Thus three fishes which had been acclimatized to the "cold" tank (9.7° at time of count) gave a mean rate of 46 respirations per minute, as compared with three "warm" fishes (33°), which gave a mean of 96. The periods of residence in the two tanks had been 120 and 61 days, respectively. In another case, 6 fishes each of "cold" and "warm" history (131 and 72 days), tested in their own tanks (11° ± and 32° ±), gave means of 46 and 89, respectively. Our results as a whole show that susceptibility to urethane and to various other drugs is a function of the metabolic rate, as manifested both by oxygen consumption and by visible respiratory movements.

In by far the greater number of our experiments the fishes, after acclimatization to the various temperatures, were subjected to the anesthetic or lethal agent at a common temperature. For the most part, this common temperature was an intermediate one, usually the air temperature of the room.

In the numerous experiments in which the test was made immediately or shortly after the transfer of the fishes to a common temperature, and in which the animals were subjected to urethane, the results were uniformly consistent in their essential features. With scarcely an exception, fishes of "warm" history outlasted those of "cold" history. In other words, fishes transferred from a higher to a lower temperature resisted the effects of the anesthetic longer (usually much longer) than those transferred from a lower to a higher temperature. For the most part, fishes of "intermediate" temperature history proved to be intermediate in their susceptibility to the anesthetic, though this relation was not as constant as that between "cold" and "warm" specimens.

A number of experiments were performed in which the fishes were kept for varying periods in water of intermediate temperature between their removal from the "cold" and "warm" tanks and the test of susceptibility. The persistence of wide differences in oxygen consumption, 48 hours (±) after the discontinuance of the temperature differ-

ences, has been discussed by Wells (1935b). The 18 fishes which were employed for this purpose in his "experiment A" were subjected to urethane at the close of the latter experiment. The "cold" lot succumbed within periods of from 5 to 55 minutes, the mean being 38. Only one of the "warm" lot, on the other hand, had succumbed by the close of 55 minutes, when the experiment was discontinued.

In the foregoing experiment, the fishes had previously been subjected to the temperature extremes for a period of 17 days. In another experiment, the periods of acclimatization were 118 days (cold) and 59 days (warm), and the interval between removal from these temperatures and the test of susceptibility was 14 days. The mean periods required for the anesthesia of the 9 fishes of each lot were 74 minutes ("cold") and 83 minutes ("warm"). This was not a very wide difference, particularly as there was considerable overlap between the periods of resistance of the two lots. A more striking difference was shown in their mean respiratory rates, these being 102 respirations per minute for the "cold" lot and 54 for the "warm." After 28 days at a common temperature, these same fishes[4] showed virtually identical respiratory rates, while their relative susceptibility to urethane had undergone a slight reversal.

The most serious defect in our experimental data is the inadequate basis which they afford for a comparison of the effects of long and short periods of subjection to temperature differences. We have very few data which permit of direct comparisons between fishes having identical history except for the length of this period. A few experiments were, however, undertaken for the purpose of comparing the reactions of fishes which had been acclimatized to warm water for 2 days with those of fishes which had been kept for much longer periods (8 and 35 days) at the high temperature. The latter (comprising both *Fundulus* and *Gillichthys*) showed a somewhat higher resistance to urethane than the former when tested at this same temperature. But the differences were not great and the numbers of individuals were so small that no great stress can be laid upon these results.[5]

On the other hand, it is interesting to note that in four experiments with *Fundulus* in which the period of acclimatization was only 24 hours, the mean figures for the "cold," "intermediate," and "warm" lots (4 of each) were approximately 20, 24, and 76 (+) minutes.[6] Taking

[4] Except that two of the "cold" lot had succumbed to the first treatment with urethane.

[5] An obvious procedure would be to test the same lot of fishes after varying intervals, and such experiments, indeed, were made. But we found some evidence that resistance to urethane increased with successive trials.

[6] This figure is too low since some of the experiments were discontinued before the "warm" individuals had completely succumbed.

these figures at face value, the difference between these "cold" and "warm" lots was even greater for these 24-hour fishes than for many of those which had been acclimatized for much longer periods. And again with gobies, it happens that the highest figure of all (135 +) is that recorded for a fish which had been kept only one day in the warm tank.

Furthermore, in two experiments with fishes (*Fundulus*) which had undergone acclimatization for only two days, the 5 "warm" fishes displayed a markedly higher resistance than the 5 "cold" ones, even after being kept from 20 hours to 2 days at a common temperature.

Despite the paucity of these last data, therefore, it seems evident that considerable and rather persistent differences in susceptibility to urethane may be brought about in these fishes in one or two days' exposure to the differing temperatures here employed.

Several experiments with chloretone in which gobies (12 fishes) were used, gave results quite in harmony with those from urethane. Large and consistent differences were shown between the fishes of "warm" and "cold" history which had been kept in their respective tanks for 2 to 3 and 4 to 5 months, respectively, and tested immediately after removal at an intermediate temperature.

Experiments with ether were unsatisfactory, since it was not easy to distinguish the point of complete anesthesia. In only one of four experiments was a significant difference noted, this being in the same direction as in the case of the anesthetics previously considered.

Our experiments with cyanides are more nearly comparable with the experiments of some of those who have worked in this field upon invertebrates. It did not, however, seem a foregone conclusion that experiments upon fishes would yield results parallel to ones upon such simple organisms as planarians. The toxicity of CN to a fish might depend upon factors other than its effect upon the utilization of oxygen. Indeed, our first experiments of this sort led us to doubt whether susceptibility to cyanide, in these animals, bore any relation to metabolic rate.

In the first five of these experiments, 0.001 M solutions of NaCN in sea water were used. The fishes (gobies) had been exposed to the differing temperatures for relatively brief periods (3 to 15 days). In four of these experiments, which were conducted at "intermediate" temperatures (20° to 22°), no significant differences were noted in the survival time of the different lots of fishes. In a fifth experiment, however, in which "intermediate" and "warm" fishes were subjected to the cyanide solution at 31° (the temperature of the "warm" water at the time), the mean survival times for the two lots of three fishes each were: "intermediate," 12 minutes; "warm," 33 minutes.

The remaining 7 experiments with cyanide differed from the preceding ones in that the periods of acclimatization were far greater, being 119 to 131 days for the "cold" fishes and 60 to 87 days for the "warm" ones. KCN was used in a 1/3,000 M solution in sea water. In 3 of these experiments, the fishes were taken directly from the temperature tanks, or the tests were made after an interval of 3 hours or less after removal. In the other 4, they were kept at a common temperature for one to two days before being subjected to cyanide. In all cases, the tests were made at "intermediate" temperatures (probably always between 18° and 20°).

Five of these 7 experiments permit of comparison between fishes of "cold" and "warm" history (7 individuals of each). The mean survival period for the former was approximately 79 minutes, that for the latter was well over 123 minutes (see foot-note 6). In two experiments in which "intermediate" and "warm" fishes were compared, the figures were about 96 and 160 minutes, respectively.

It is of interest that whereas the maximum difference in susceptibility to urethane (at a common temperature) was nearly or quite reached after a period of a day or two at the contrasted temperatures, conclusive differences in susceptibility to cyanide were not revealed until a much longer period had elapsed. Furthermore, experiments with de-oxygenated water gave results similar to those with cyanide.

In these experiments, the fishes were placed in 500-cc. flasks, filled with sea water which had been boiled or subjected to evacuation with an air-pump. Care was taken that air bubbles should be excluded and that all flasks used in any one experiment should be maintained at the same temperature.

In four experiments with *Fundulus,* the results of two in which the fishes had been acclimatized for 35 days were in conformity with those of the cyanide experiments with gobies in which long-time acclimatization had been practised. In the other two, in which the fishes had been subjected to temperature differences for 2 or 3 days, the "warm" fishes actually succumbed before either the "cold" or "intermediate" ones.

Two experiments with *Gillichthys* (6 fishes from each tank), in which the acclimatization periods were relatively brief (8 to 14 days), gave contradictory results. The mean survival times for the two in minutes were: "cold," 161; "intermediate," 178; "warm," 154.

In three experiments, on the other hand (7 "cold," 7 "warm" fishes) in which the acclimatization periods were much longer (62 to 87 days for "cold," 121 to 146 for "warm"), the mean survival times were 110 and 145 minutes, respectively. In another experiment, furthermore, in which 2 "intermediate" and 2 "warm" fishes were com-

pared, the mean figure for the former was 137, that for the latter, 210. It may be added that in three of the foregoing experiments in which the matter was tested, all of the "warm" fishes recovered when returned to running sea water, while none of the "cold" or "intermediate" fishes did so.

Thus, as in the case of the experiments with cyanide, fishes which had been subjected to the temperature differences for considerable periods (one to several months) showed marked differences in their powers of resistance, while those subjected for much briefer periods (two weeks or less) gave results which were indecisive or mutually contradictory.

One extremely instructive experiment was performed in which heat was employed as a lethal agent. Five "cold," 5 "intermediate," and 4 "warm" gobies were used (the entire available stock at the time). The first had been in the "cold" tank for 146 days, the last in the "warm" tank for 87 days, while the "intermediate" ones had been kept for many months in natural sea water, subject only to seasonal changes of temperature (18.5° ± at the time of this experiment). The high and low temperatures were discontinued, and the fishes brought to a common temperature (18.5°) two days before the test of endurance to heat was commenced. For the latter purpose, the temperature to which all the fishes were subjected was rapidly raised, so that it reached 33° in 45 minutes. The five fishes of "cold" history succumbed almost simultaneously in 67 to 69 minutes, the temperature being 35.6° at the time. Those of "intermediate" history all succumbed (also simultaneously) in 106 minutes, the temperature having risen to 38°. The four fishes of warm history were living one and a half hours later, by which time the temperature had risen to 39.3°. This was then lowered somewhat, falling to a minimum of 36° during the night. Upon being raised to the same maximum in the morning, the fishes died within another half hour.

From the foregoing experiments the inference seemed warranted that differences in the temperature history of the fishes concerned resulted in differences in metabolic rate, which manifested themselves both in the visible rate of respiration of these fishes and in their susceptibility to certain anesthetics and lethal agents. It seemed worth while, accordingly, to test the effect of some drug which was known to accelerate oxidative metabolism in other animals. For this purpose, 2, 4-dinitrophenol was used in a concentration of 2 per cent of a saturated solution in sea water.[7]

[7] This substance is but slightly soluble in water. Even a saturated solution is extremely dilute.

Gobies which had been kept in running sea water (about 19° at the time) were used for these tests. Without describing these experiments in detail, it may be said that fishes kept for 16 hours in the d-n-p solution were markedly more susceptible to urethane than controls taken from pure sea water. Since, however, the d-n-p had a somewhat toxic effect, even at this low concentration, it is far from certain that the greater susceptibility of the fishes thus treated was due to heightened metabolism.

Summary and Conclusions

The experiments herein discussed were made upon two species of teleost fishes, *Fundulus parvipinnis,* and *Gillichthys mirabilis.* The anesthetics used were urethane, chloretone, and ether, the lethal agents NaCN, KCN, asphyxiation, and excessive temperature.

Fishes which had been kept at widely different temperatures, when tested at these temperatures, were found to differ markedly in the rate of their visible respiratory movements, just as they were already known to differ in their rate of oxygen consumption. Other things equal, these are higher at higher temperatures than at lower ones. Susceptibility to the single drug used in this connection (urethane) was also higher at the higher temperatures.

When fishes which had become acclimatized to high and low temperatures, respectively, were transferred to a common temperature (usually an intermediate one), those from the warmer water displayed a lower respiratory rate and a lower susceptibility to the anesthetics and lethal agents than those from the colder water. Thus the differences in physiological activity which had been originally induced by acclimatization to these differing temperatures were completely reversed when the fishes were brought to a common temperature. After this transfer, it was the former "warm" fishes which had the lower respiratory rate, etc., and the former "cold" fishes which had the higher. Fishes kept at intermediate temperatures remained intermediate.

Acclimatization to a high temperature appears to consist, in part at least, of a change whereby an initial great increase in metabolic rate is followed by a regulative process, with continued sojourn in warm water, and, conversely, that acclimatization to a low temperature involves a similar process working in the opposite direction.

While the degree of acclimatization must be, within limits, a function of time, it is surprising that after a period of one day (possibly even less) at contrasted temperatures, the maximum difference in susceptibility to urethane, when determined at a common temperature, seems to have been nearly or quite reached. When cyanides and oxy-

gen-free water were used, on the other hand, conclusive differencs were not revealed until after a month or more of acclimatization. It may be that susceptibility to these different agents rests upon a somewhat different basis.

It is likewise noteworthy that the induced differences persisted, in one case at least, as much as 14 days after withdrawal of the environmental cause.

Rather limited experiments with dinitrophenol, a substance known to increase the metabolic rate of some animals, showed that this drug brought about a considerably increased susceptibility to urethane. It is not certain, however, that this result was due merely to an increase in metabolic rate.

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THE MELANOPHORE-DISPERSING PRINCIPLE IN THE HYPOPHYSIS OF FUNDULUS HETEROCLITUS

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The numerous studies of the pigmentary changes of *Fundulus* made by Parker and his students at these laboratories, and similar studies on other fishes made by various European investigators (Pouchet, 1876; von Frisch, 1911) have, in the main, led to the conclusion that in the teleosts the ability of background adaptation depends on the nervous system.

The rôle of the pituitary gland in teleost color change has not yet been conclusively established. The responses of the melanophores of fishes to various commercial preparations of the pituitary gland leave much to be desired in the way of convincing and decisive results. Abolin (1925) reported dispersion of the melanophores of *Phoxinus* following injection of "infundin," a commercial preparation of the posterior lobe used in obstetrics. Hewer (1926) found that extracts of the posterior lobe of the cod pituitary gland produced the concentrated state of the melanophores in *Phoxinus* and that "infundin" gave inconclusive results. Giersberg (1930), on the other hand, was able to confirm Abolin with regard to the dispersing effect produced by "infundin." Przibram (1932) accounted for the differences between the results of Abolin and those of Hewer as due to differences in dosage. Spaeth (1918) and Wyman (1924) both found that commercial obstetrical "pituitrin" produced the concentrated condition of the melanophores in *Fundulus;* the former used isolated scales as test material and the latter made intraperitoneal injections.

Odiorne (1933), using several pituitary extracts prepared for clinical use, was unable to confirm Spaeth and Wyman. He placed isolated *Fundulus* scales in solutions of "pituitrin" but could not obtain the melanophore-concentration reported by Spaeth. Similarly, the injection of "pituitrin" intraperitoneally into dark- and light-adapted specimens produced no change in color, the results thereby differing from those of Wyman. By using "antuitrin," an extract of the anterior lobe of the hypophysis, Odiorne obtained the following interesting results: intraperitoneal injection of this extract brought about the concentrated state of the melanophores of dark-adapted *Fundulus* kept

on a black background. The response was prompt but irregular in that it did not affect all the melanophores, and lasted not more than two hours. When "antuitrin" was added to a small amount of Ringer's solution in which isolated scales had been placed, the expanded melanophores contracted within a few minutes. The results with "antuitrin" are thus similar to those obtained by Spaeth and by Wyman, who both used "pituitrin" of the same lot. Odiorne therefore concluded that Spaeth, in presenting a new method for the standardization of pituitary extracts, was in truth doing little more than "measuring their impurity with regard to material from the anterior lobe."

There are a number of criticisms to be made of these investigations with pharmaceutical preparations. First, the use of commercial extracts of the posterior pituitary is to be discounted in the delicate qualitative tests of pigmentary reactions because such preparations probably contain the chromatophorotropic hormone only as an impurity from the intermediate lobe. It has been conclusively demonstrated from the work of Smith (1916), Allen (1916), Atwell (1919, 1934), and Swingle (1921), that the intermediate lobe of the pituitary is the localized region for the production of the melanophore-stimulating hormone in amphibia. Noble and Bradley (1933) have similarly shown that lizards, completely deprived of their intermediate lobes, are incapable of color changes. Zondek and Krohn in a series of papers in 1932 reported the isolation of "intermedin," the specific chromatophore-activating hormone, from the intermediate lobes of the pituitaries of several animals. It is probable that commercial preparations of the posterior lobe vary in their content of the chromatophorotropic hormone according to the methods of extraction and purification used.

Secondly, the solvent media and preservatives employed in these preparations may affect melanophore activity. For example, the Parke, Davis and Company obstetrical "pituitrin" is made up in 0.5 per cent chloretone. Adequate control experiments for some of the above investigations should have been made with similar injections of chloretone rather than with Ringer's solution, especially since Wyman reported that chloretone caused a dispersion of melanin granules in the pigment cells of *Fundulus*. "The reaction (of melanophores) can also be produced by various non-specific substances contained in organ-extracts, and by quinine, curarine, choline, acetylcholine, caffeine, and various preservatives" (Zondek, 1935).

Thirdly, the use of isolated scales as test objects is not a satisfactory method. The results obtained from such experiments do not

necessarily imply that the mechanisms involved in the melanophore changes of the isolated scales also apply to the intact animal. Additional factors which may play a significant part in the reactions of isolated melanophores are slight salt impurities in the Ringer's solution, hypo- or hypertonicity, and oxygen deficiency of the solution in which the scale is immersed.

The treatment with pituitary extracts reviewed in the above studies is thus seen to offer no decisive conclusion as to the part played by this hormone in the color changes of *Fundulus*. The alternate approach to this problem is that involving ablation of the hypophysis. Matthews (1933) successfully hypophysectomized *Fundulus* and noted that this operation had no effect on the pigmentary responses, since such operated fishes underwent normal color changes. However, the pigment granules in the melanophores became *concentrated* when he immersed an isolated scale in an extract of one *Fundulus* pituitary ground up in 0.05 cc. of N/10 NaCl. From this Matthews concluded that the hypophysis of *Fundulus* does contain the melanophore-activating principle.

In view of the criticisms made of the methods involving the use of isolated scales, it was thought advisable to test the chromatophorotropic activity of *Fundulus* pituitaries on a number of animals which are known to have definite melanophore responses to this hormone. This problem was undertaken at the suggestion of Professor Alden B. Dawson to whom I am indebted for many helpful criticisms and kind suggestions. Dr. A. A. Abramowitz also very kindly gave of his time and advice.

Materials and Methods

The animals used as test objects in this investigation were *Fundulus heteroclitus* (the common killifish), *Ameiurus nebulosus* (catfish), *Rana pipiens*, and *Anolis carolinensis* (the American "chameleon"). Extracts of the *Fundulus* pituitaries were made by grinding the glands with a glass rod in a Syracuse dish the bottom of which had been roughened with powdered emery. Solutions of different concentrations were made of these glands in cold-blooded Ringer's and were injected intraperitoneally.

The normal color reactions of the test animals are considered under each sub-title.

Anolis carolinensis

This lizard is the so-called "chameleon" found in our southern states. Specimens obtained from a dealer in Georgia were caged and

kept in a warm room. Feeding with blow-flies and daily sprinkling of the cages with water maintained the animals in excellent condition.

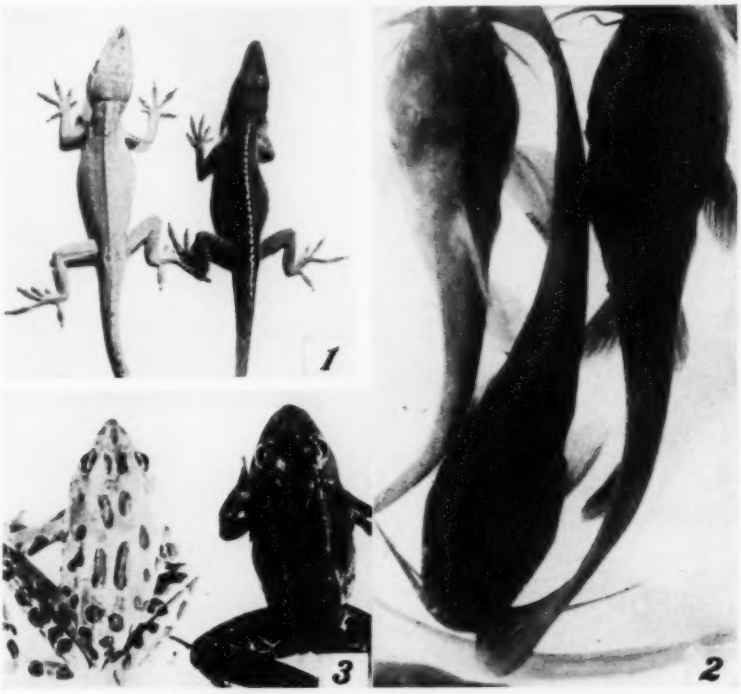
These lizards are brown at room temperature (18°–28° C.) in the light, because the melanophores are in the dispersed condition. In darkness the animals are green because the melanin granules have retracted from immediately beneath the epidermis into the primary and larger branches of the melanophores, thus exposing the yellow oil-droplet layer; light, which is reflected from the leucophore layer as blue, mixes with the yellow rays from the oil-droplet layer and so appears green at the surface (von Geldern, 1921). If dark animals are placed upon a brightly-illuminated white background they gradually turn green. The time for this change requires from 10 to 30 minutes, but varies with different specimens. Conversely, a light animal placed on an illuminated black background darkens, but the reaction is more rapid, and takes place in from 5 to 15 minutes. Ablation of the anterior lobe of the pituitary does not interfere with the normal color changes, but those specimens from which the intermediate lobes have also been removed lose the ability to darken on backgrounds which normally evoke the dark condition, and remain permanently in the green state. Fig. 1 shows the striking difference in color between a normal and an hypophysectomized specimen.

Three normal lizards were placed in the dark-room for an hour. At the end of this time they were green in color. Two were each injected with 0.25 cc. of an extract of 4 *Fundulus* pituitaries in 0.5 cc. of Ringer's; the third animal was injected with 0.25 cc. of brain tissue suspension. The following table shows the results:

	Pituitary	Pituitary	Brain
At injection	Green	Green	Green
20 minutes after	Brown	Brownish-green	Green
45 minutes after	Brownish-green	Brownish-green	Green
60 minutes after	Brownish-green	Green	Green
80 minutes after	Green	Green	Green

The two animals injected with the pituitary extract darkened, whereas the control remained unaffected. The rapid fading of the induced dark condition was due to the small amount of pituitary tissue injected. When 8 *Fundulus* pituitaries in 0.4 cc. of Ringer's were injected into an animal that had been adapted to darkness for an hour and was consequently in the green condition, the brown color response was obtained in 12 minutes and persisted, even though the animal remained in the dark-room, for over 6 hours.

One hypophysectomized lizard which had remained green for more than 10 weeks was injected with 0.25 cc. of 4 *Fundulus* pituitaries in 0.30 cc. of Ringer's. The animal turned brown after 3 minutes and remained dark for more than 12 hours, having been kept all the time on a brightly illuminated white background.



EXPLANATION OF PLATE I

FIG. 1. On the right is a normal *Anolis* in the dark condition. The animal on the left has been hypophysectomized and is consequently in the green or light state.

FIG. 2. On the right is a normal catfish that has been darkened by adaptation to a black background. The fish in the center had been light-adapted to a white background and then been injected with 12 *Fundulus* pituitaries; one hour after the injection it was photographed in this dark condition. The animal on the left is a control light-adapted fish that had received 0.5 cc. of Ringer's solution intraperitoneally.

FIG. 3. The frog on the right had been light-adapted and then injected with 8 *Fundulus* pituitaries, photographed 75 minutes after the injection. The animal on the left was the control that received an injection of Ringer's solution.

Two specimens, adapted to darkness, were injected each with 0.20 cc. of Ringer's solution. They showed no color change.

Rana pipiens

Four frogs were placed on an illuminated white background for 24 to 48 hours. At the end of this time they were maximally pale, the web melanophores being in the punctate condition. Three of these frogs were injected with extracts of *Fundulus* pituitaries, so that each received the equivalent of 8 fish glands; the fourth animal was injected with Ringer's solution. The animals which received the pituitary began to darken within 15 minutes and after an hour were extremely dark, the web melanophores being reticulate (Fig. 3). The dark condition persisted for 3 to 4 hours although the animals remained on the illuminated white background. The control specimen showed no change in color.

Ameiurus nebulosus

These fishes become light greenish on a white background and very dark brown or black on black backgrounds. Of three animals adapted to a white background, one was injected with 8, and a second with 12 macerated *Fundulus* pituitaries, while the third received 0.5 cc. of Ringer's solution. The fishes receiving the pituitary injections began to darken slightly after 30 minutes and at the end of one hour were perceptibly much darker than the control (Fig. 2). In both animals

EXPLANATION OF PLATE II

The photographs on this plate were very kindly taken for me by Mr. E. P. Little, on the staff of the Marine Biological Laboratory.

FIG. 4. The band in the caudal fin of a *Fundulus;* this region has faded, 9 days after the denervating cut, as a result of keeping the fish in an illuminated white dish. The original band is shown outlined in ink.

FIG. 5. Detail of a region in the band of Fig. 4, showing the melanophores in the punctate condition.

FIG. 6. Darkening of a faded denervated band induced by the injection of *R. pipiens* pituitary tissue.

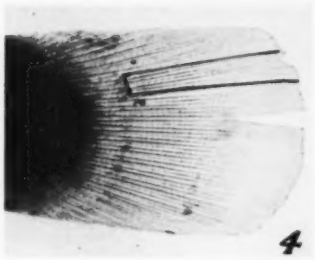
FIG. 7. Detail of the band in Fig. 6, showing the melanophores in varying degrees of dispersion. At the top of the photograph is shown an innervated region of the tail with the melanophores punctate.

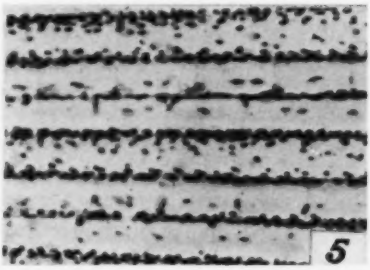
FIG. 8. Slight darkening of the faded band resulting from the injection of *Fundulus* pituitaries.

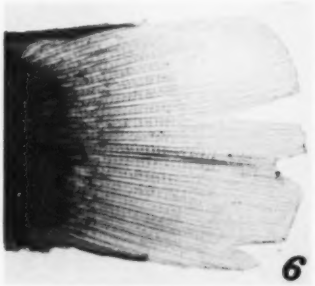
FIG. 9. Detail of a portion of the band in Fig. 8. Some of the melanophores show stellation.

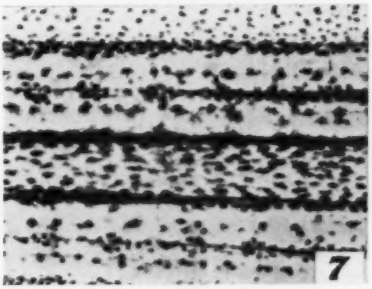
FIG. 10. A darkening of the band provoked by the injection of Parke, Davis and Company obstetrical "pituitrin."

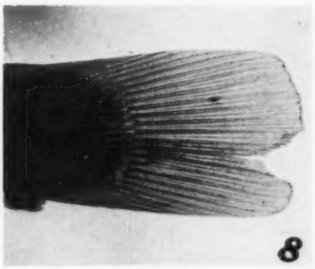
FIG. 11. Low power microscopic view of a portion of the band in Fig. 10, showing the extreme dispersion of the melanophores. At the bottom of the print is a portion of an innervated region showing the melanophores in the punctate condition

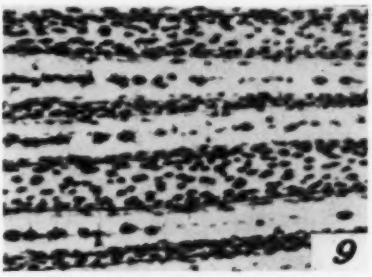


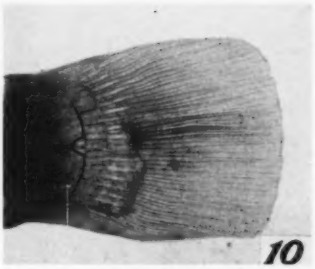












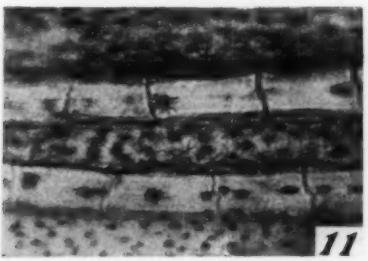


PLATE II

thus treated, however, the dark condition attained was not as pronounced as that obtained normally by adaptation to a black background. The control catfish remained in the light condition.

Fundulus heteroclitus

Specimens were placed in white or black dishes and after 24 hours were fully adapted to their backgrounds, the animals in the white dish being light, and those in the black dish, dark. In the first test, one dark and one light fish each received 0.25 cc. of extract of 2 *Fundulus* pituitaries in 0.5 cc. of Ringer's. The fishes showed no color changes for two hours.

In a second test, a suspension of 5 *Fundulus* pituitaries was injected into a light killifish and a similar solution into a dark fish. There was no color change in either during three hours of observation.

In the third test, the equivalent of 10 hypophyses was introduced into a light and into a dark *Fundulus*. For the following 90 minutes the melanophores remained unaffected by this concentrated extract.

The work of Matthews shows that ablation of the hypophysis does not affect the normal color changes of *Fundulus*. He believed that the pituitary of this fish did contain the melanophore-activating principle since it caused a *concentration* of the melanin in the pigment cells of an isolated scale that was immersed in an extract of the gland. The fact that the melanophores of those vertebrates, which respond to the pituitary pigmentary hormone, do so by a *dispersion* of the melanin granules, would imply either that the melanophore reactions of *Fundulus* differ from those of other vertebrates or that the isolated scale technique is at fault.

Although hypophyseal extracts from *Fundulus heteroclitus* are capable of dispersing the pigment within the melanophores of a fish, an amphibian, and a reptile, the melanophores of *Fundulus* itself remain unaffected by such extracts. Both Hewer and Matthews have offered the suggestion that such lack of response might be due to the secretion of sub-minimal amounts of the pigmentary hormone in the normal animal. I have found relatively concentrated extracts of 5 or 10 glands to have no observable effect on the melanophores.

The possibility appeared that the failure of the melanophores of *Fundulus* to respond to its own demonstrably-active pituitary gland might be due to some masking action of the nervous system. To test this, denervated bands were made in the caudal fins of *Fundulus* according to the method used by Wyman (1924). This consists of making a transverse incision across the fin rays from one surface of the tail to the other surface. As a result, the radiating nerve fibers

of that region are cut and a denervated band of melanophores is formed extending from the cut distally to the end of the caudal fin. The pigment in the area distal to the cut becomes maximally dispersed within several minutes. Parker (1934*a*) attributes the darkening of this band to the mechanical stimulation of the dispersing nerve fibers at the time of making the incision. Degeneration of the nerves proceeds posteriorly from the incision and regeneration from the nerves anterior to the cut does not begin until about 18–20 days after the initial operation (Parker and Porter, 1933). There is thus a period of several days when the melanophores in such bands are without physiological nerve supply.

Three specimens of *Fundulus* with prepared denervated bands were placed in white dishes. When examined on the ninth day after the operation the bands were completely faded, the melanophores in these areas being punctate, as were also the melanophores in the unoperated regions of the fishes (Figs. 4 and 5). Into each of these animals was injected a suspension of 5 neuro-intermediate lobes of frog hypophyses in Ringer's solution. The bands darkened in from 10 to 20 minutes; microscopic examination showed that the melanophores in these areas had their pigment dispersed, some to the maximum amount and others to a stellate condition (Figs. 6 and 7). The melanophores in the innervated regions of the caudal fins remained punctate.

This experiment was repeated with extracts prepared from the pituitary glands of *Fundulus*. The procedure was to note the condition of the melanophores in a definite region of the band, make the injection, immediately afterwards return the animal to its illuminated white dish, and at varying intervals to examine the same region microscopically. The protocols for 5 experimental fishes best describe the results:

Animal No. 2.

June 22. Caudal band denervated. Placed in white dish.

July 2. Band has faded.

10:40 A.M.—most of the melanophores in the band are punctate, some are very slightly stellate.
Injected 0.20 cc. of 5 *Fundulus* pituitaries in 0.25 cc. of Ringer's.

11:00 A.M.—band has darkened; the melanophores in the denervated area are pronouncedly stellate; those in the innervated areas are punctate.

Animal No. 6.

June 24. Female, band prepared. Placed in white dish.

July 4. 11:30 A.M.—band completely faded, melanophores punctate.

11:34 A.M.—injected 0.20 cc. of 8 *Fundulus* pituitaries in 0.25 cc. of Ringer's.

11:45 A.M.—band darkening, many melanophores show beginning stellation.

11:55 A.M.—most of the melanophores are slightly stellate; a few are completely dispersed.

Animal No. 7.
June 24. Male, operated and placed in white dish.
July 5. 3:44 P.M.—band faded, melanophores punctate. Injected 0.20 cc. of 8 *Fundulus* pituitaries in 0.25 cc. of Ringer's.
3:55 P.M.—no change.
4:05 P.M.—no change.
4:15 P.M.—no change.

Animal No. 8.
June 27. Female, operated and placed in white dish.
July 5. 10:05 A.M.—band faded, melanophores punctate.
10:07 A.M.—injected 0.15 cc. of 4 *Fundulus* glands in 0.20 cc. of Ringer's.
10:20 A.M.—slight initial stellation in the band.
10:30 A.M.—some of the melanophores are slightly stellate.

Animal No. 10.
June 27. Female, band denervated, placed in white dish.
July 10. 11:33 A.M.—band faded, melanophores punctate.
11:35 A.M.—injected 0.13 cc. of 8 *Fundulus* pituitaries in 0.15 cc. of Ringer's.
11:42 A.M.—band darkening, melanophores of the band are slightly stellate.
11:55 A.M.—band dark; a few of the melanophores are completely dispersed, many show slight stellation, and some remained punctate.

In these tests, denervated melanophores responded to pituitary injections by a dispersion of their pigment granules (Figs. 8 and 9). Not all of the melanophores in the band reacted to this substance, and not all were affected to the same extent.

In a third series of tests, five *Fundulus heteroclitus* females with faded denervated bands (9 and 10 days after the denervating cuts) were each injected with 0.20 cc. of Parke, Davis and Company obstetrical "pituitrin" (ampoules no. 3018380 and no. 3064766). The bands darkened after 10 minutes and microscopic examination showed the melanophores of those regions to be in a condition of extreme dispersion (Figs. 10 and 11). As Odiorne has previously reported (1933), the "pituitrin" had no effect on the remaining innervated melanophores of the tail and body. Three of these injected animals were kept under observation for two hours. At the end of this time the band of one fish had faded only very slightly while those of the remaining two specimens persisted unfaded. Because this pituitary solution contains 0.5 per cent chloretone, added as a preservative, controls were run on three fishes possessing faded caudal bands. Intraperitoneal injections of 0.20 cc. of 0.5 per cent chloretone dissolved in amphibian Ringer's solution were made. Neither the denervated bands nor the innervated melanophores showed any change, all remaining in the punctate condition. Wyman (1924) found that when a 1 per cent chloretone solution was siphoned over the gills of *Fundulus*, the inner-

vated melanophores very soon became dispersed, while the melanophores in the faded caudal bands remained punctate or slightly stellate. The discrepancy between Wyman's and my results may be due to a rapid elimination or destruction of the drug by the body fluids.

The response of denervated melanophores to hypophyseal extracts suggests that the pituitary secretion of *Fundulus* may be a humoral factor to be considered in those studies of color changes where the method of denervated regions is used. In this connection, the study made by Parker (1934*b*) on the catfish, *Ameiurus*, is of interest. He found that the pituitary gland of this fish contains the melanophore-dispersing principle. Hypophysectomized catfishes, however, undergo the same color changes on black or white backgrounds that normal unoperated specimens do. When a catfish in the light state with a faded denervated band is put into a black dish, the light band becomes dark approximately at the same time as the whole fish does. When light fishes with faded bands are hypophysectomized and then placed in black dishes, the bands remain light for several hours, after which they darken. This difference in the darkening of the denervated bands Parker attributes to the pituitary hormone in the blood of the normal catfish and to its absence from the hypophysectomized animal. The final darkening of the band in the operated fishes he believes due to the diffusion into the band of a dispersing neurohumor from the adjacent nerve terminals. Whether *Fundulus heteroclitus* will react in similar fashion to hypophysectomy remains to be investigated.

Summary

1. From these experiments it appears that the hypophysis of *Fundulus heteroclitus* contains a hormone which is capable of dispersing the melanin granules in the pigment cells of the catfish, the frog, and the lizard.

2. The innervated melanophores of *Fundulus* are unresponsive to the pigmentary hormone of their own pituitary glands, even though concentrated extracts were injected, and are also unresponsive to extracts from amphibian and mammalian pituitaries.

3. This lack of response of the normal innervated melanophores appears to be due to some nervous control, because denervated melanophores respond to pituitary injections by a typical *dispersion* of their pigment. This result is in contrast to that reported by Matthews, who used isolated scales.

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RENAL FUNCTION IN MARINE TELEOSTS
I. Urine Flow and Urinary Chloride

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Studies on renal function in the fishes have brought out some exceedingly interesting facts which have been of considerable value in the general problem of kidney function (for review of the literature see Marshall, 1934) and justify a more extended study along these lines. As a necessary basis for further physiological work, it was essential to obtain information concerning the normal rate of urine flow in the marine teleosts. The reports available in the literature were highly variable (Burian, 1909; Edwards and Condorelli, 1928; Marshall, 1930; Smith, 1930); and the theoretical considerations of Smith (1930) demanded a very low urine flow in marine teleosts as compared to fresh-water species.

In a previous investigation (Grafflin, 1931) we were led to the conclusion that the normal urine flow of the glomerular sculpin (*Myoxocephalus octodecimspinosus*) is probably below 4.0 cc., and of the aglomerular toadfish (*Opsanus tau*) is probably below 2.5 cc. per kilogram per 24 hours. It was further concluded that the normal urine of both species is almost invariably free of chloride and relatively high in total nitrogen. When these animals were followed under the usual experimental conditions, they were found to develop a marked diuresis, with the appearance of large quantities of chloride in the urine, a rapid diminution in the urinary total nitrogen, and an elevation of the plasma chloride. With the use of extreme precautions to avoid skin injury, it was possible to obtain low urine flows and zero (or trace) urinary chlorides in the majority of animals. Some animals with low flows, however, showed a rather high concentration of urinary chloride; while a number of animals showed a relatively high rate of flow, which was sometimes associated with a fairly high urinary chloride, but at other times with a low, or even zero, chloride.

[1] Some of the data reported in this paper were obtained while I was a Fellow of the John Simon Guggenheim Memorial Foundation (1934). I wish to express my gratitude to Prof. R. Dohrn, Director of the Stazione Zoologica, Naples, Italy, and to Dr. E. J. Allen, Director of the Laboratory of the Marine Biological Association, Plymouth, England, and their staffs for their generous hospitality and coöperation while I was a guest at their laboratories.

These latter findings led us to seek additional data upon the normal rate of urine flow, and to attempt to settle (1) whether there is any definite correlation between the rate of urine flow and the urinary concentration of chloride; and (2) whether the urinary excretion of chloride in the marine teleosts may be considered a normal physiological process, or whether it is invariably to be interpreted as a criterion of abnormality. In the experiments upon urine flow, the length of the urine collection period was varied in an attempt to determine whether some factor such as an initial diuresis or anuria following catching and tying of the urinary papilla was confusing the results.

Material and Methods

The fishes used in this study were the sculpin (*Myoxocephalus octodecimspinosus*), flounder (*Pseudopleuronectes americanus*), cod (*Gadus morrhua*), goosefish (*Lophius piscatorius*), plaice (*Pleuronectes platessa*), *Orthagoriscus mola*, *Scorpæna scrofa*, *Trigla corax*, and *Zeus faber*. The sculpins and flounders [2] recorded in Figs. 1 and 2 were handled in the following manner. Immediately after being hooked each animal was brought to the surface and dipped up in a bucket. The hook was removed under water, the fingers touching only the inside of the mouth, and any fish bleeding and (or) torn at the point of hooking was discarded. The fish was then transferred without touching to a shallow porcelain bowl, where it was held under water with bare hands, and with great care to avoid skin injury and interference with respiration. The bladder was emptied by massage of the overlying abdominal wall and the urinary papilla was tied off with fine silk thread. Catheterization was not used in the preliminary emptying of the bladder for the following reasons. The urinary papilla is extremely sensitive, and insertion of the catheter frequently causes the animal to struggle vigorously, with increased skin injury as the inevitable result. Furthermore, it was desirable to eliminate any possible reflex effects which the stimulus of catheterization might have upon the kidney. At the end of the tying period the animal was killed by a blow on the head and the accumulated urine was removed from the exposed bladder by syringe. The animals were then weighed, and urine flows are expressed in terms of cc. per kilogram of fish per 24 hours. Chloride (expressed as millimols per liter) was determined by the method of Van Slyke (1923) with the modifications reported by Smith (1930). The experiments upon sculpin and flounder were carried out at the Mount Desert Island Biological Laboratory, Salsbury Cove, Maine.

[2] The kidneys of both species are glomerular (Nash, 1931; Marshall and Grafflin, 1932; Marshall, 1934; Clarke, 1934).

Results

Sculpin [3]

The data on 83 sculpins are summarized in Fig. 1, and show a considerable variation in urine flow. Since failure to empty the bladder completely at the beginning could lead to serious error, particularly in short collection periods, we tested our ability in this regard on 22 freshly caught sculpins handled with the same precautions as the experimental animals. The residues were as follows: 8—completely empty; 11—0.02 to 0.03 cc.; 1—0.05 cc.; 1—0.32 cc.; 1—0.45 cc. In the last two animals the recorded urine flows would have been too high by about 9 cc. in 6 hours, 2.3 cc. in 24 hours. With a residue of 0.03 cc. the error would be, for a fish of 175 grams (average of series), 0.7 cc. for 6 hours, 0.17 cc. for 24 hours. The danger of incomplete emptying is further emphasized by one group of four sculpins which were emptied in the same manner by another person, and in which the residual urine on immediate sacrifice would have introduced errors of 2.8, 4.0, 4.4 and 12.0 cc. in a 6-hour period, 0.7 1.0, 1.1 and 3.0 cc. in a 24-hour period.

It is clear that incomplete emptying of the bladder at the beginning of the collection period must be regarded as a serious source of error in the sculpin. The aberrant high flows in Fig. 1 could all be easily explained on this basis, and it is certain that many of the other flows are too high, some of them by a considerable margin. Nevertheless, in view of the results obtained on the control animals, when this source of error is fully taken into account it must still be concluded that there is a marked variation in the *actual* urine flow under our experimental conditions. The relatively narrow range of variation in the longer collection periods (over 30 hours) is deceptive, since some animals tied up for such a long period showed over-distention of the bladder, and had to be discarded. The data on relatively short collection periods (6.5–8.5 hours) are particularly interesting in view of the following statement by Clarke and Smith (1932): "There is evidence in our experiments that (reflex?) anuria may occur in fish and that it may persist for some hours, particularly after the fish is first catheterized. This fact is of great importance in the measurement of 'normal' urine flows and has been overlooked by investigators in the past (compare Smith, 1930; Grafflin, 1931)" (p. 142). An examination of Fig. 1 makes it quite clear that such a period of anuria did not occur in our experimental animals. Actually, the data strongly suggest a signifi-

[3] I wish to express my indebtedness to Mr. David Ennis for his assistance in the sculpin and flounder experiments recorded in Figs. 1 and 2.

cantly higher average rate of urine flow in the hours immediately following emptying and tying. However, we feel that without additional data the indicated "initial diuresis" cannot for the present be accepted as proved.

The urinary chloride concentrations in the same series of 83 sculpins are summarized in Fig. 2. The chloride is from 0 to 10 millimols per liter in over half of the animals (46) and is below 50 millimols per liter in over two-thirds (58). The significant fact is that the 37 sculpins showing more than a trace of chloride (over 10 millimols per liter) are scattered indiscriminately over the entire range of urine flow.

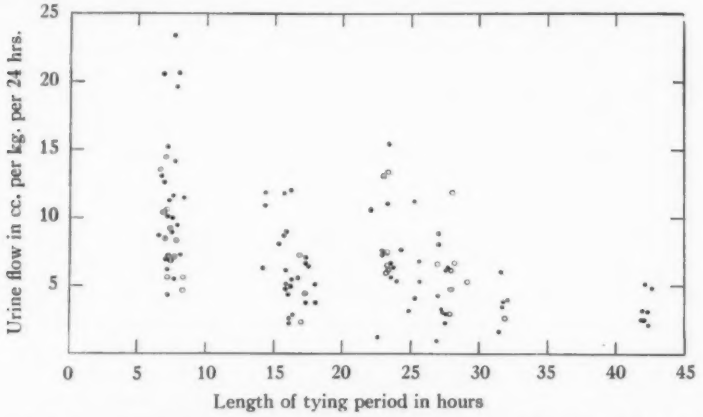

FIG. 1. Observations upon rate of urine flow in freshly-caught sculpins and flounders, with urine collection periods of variable length. Solid circles, Sculpin. Open circles, flounder.

Discussion of Sculpin Data and Conclusions

The two most important facts brought out by the present data are: (1) the wide variation in the urine flow and urinary chloride concentration in freshly caught sculpins, under experimental conditions which were as ideal and as nearly constant as could be obtained; (2) the lack of any direct relationship between the rate of urine flow and the urinary chloride concentration, which is obvious from even a casual examination of Fig. 2. Grafflin (1931), from more meager data and at a time when many of the problems involved in fish experimentation were not fully appreciated, was led to the conclusion that the normal urine flow of the sculpin is probably below 4.0 cc. per kilogram per day. The present experiments do not bear out this earlier conclusion, but indicate rather a considerable range of urine flow as a normal base-line

for physiological work. The chloride data likewise question the interpretation of the presence of chloride in the urine as a criterion of abnormality, and suggest instead that urinary chloride excretion may be a normal physiological process in the sculpin. In this connection the following observations are of interest. In an earlier study (1931) we reported the chloride concentration of original urine, removed from the bladder immediately after catching with hook and line, to be zero or trace in a short series of sculpins. Pitts (1934) has more recently reported similar data upon eleven sculpins, eight of which showed zero urinary chloride, while the other three showed 53, 74 and 147 millimols per liter respectively. In four specimens of the closely related daddy

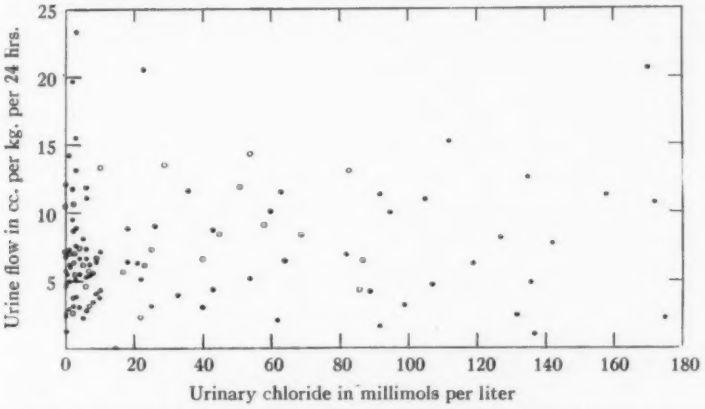

FIG. 2. Graph showing urinary chloride concentration, plotted against rate of urine flow, in the sculpins and flounders recorded in Fig. 1. Solid circles, sculpin. Open circles, flounder.

sculpin (*Myoxocephalus scorpius*) Pitts obtained values of 0, 34, 48, and 128 millimols per liter. When Pitts' positive demonstration of chloride in considerable amounts in the urine of six out of fifteen freshly caught sculpins (two species) is considered in conjunction with the present data, we feel that the conclusion is probably justified that the sculpin may excrete variable, and sometimes considerable amounts of chloride in the urine under normal physiological conditions. On this basis, concurring with the conception that the majority of sculpins normally excrete an essentially chloride-free urine, the presence of chloride in the urine cannot be accepted as a criterion of abnormality without further proof, particularly since it is often found in large amounts with a very low rate of urine flow.

Sculpins with Pyloric Tie

Smith (1930, 1932) has demonstrated quite conclusively that marine teleosts normally ingest relatively large quantities of sea water, which undergoes absorption in the gastro-intestinal tract. Grafflin and Ennis (1934) have recently reported experiments designed for the study of urine formation when this intestinal absorption of sea water was prevented. Sculpins, freshly caught and handled with care, were anesthetized with urethane and the upper intestine was tied off at its junction with the stomach. At the end of the operation, with the animals completely relaxed by the anesthetic, the bladder was emptied by massage of the overlying abdominal wall. The urine flow and urinary chloride data obtained upon these operated animals in the ensuing short collection period (5.5–9.9 hours) are particularly interesting in connection with the data reported above. In brief, the results on 24 such sculpins were as follows: in 16 the chloride varied from 0 to 9 millimols per liter, urine flow 5.4 to 19.3 cc. per kilogram per 24 hours; in the other 8, chloride from 16 to 120 millimols per liter, flow from 6.8 to 16.0 cc. Even when incomplete preliminary emptying of the bladder as a source of error is fully taken into account, these data furnish additional evidence that in the sculpin there is no direct relationship between the rate of urine flow and the urinary chloride concentration. The wide variation in the rate of urine flow in these animals, under experimental conditions which were essentially identical, is likewise noteworthy.

Flounder

The data upon urine flow and urinary chloride in thirty flounders are included in Figs. 1 and 2. As in the sculpin, incomplete emptying of the bladder at the start of the collection period is a potential source of considerable error. However, in only one of the control animals was there any measurable residue, and in that case it was 0.1 cc. When this source of error is taken fully into account, the data demonstrate a considerable variation in the actual rate of urine flow under our experimental conditions. Furthermore, the results obtained in short collection periods (6.5–8.5 hours) make it evident that in the flounder, as in the sculpin, the manipulations involved in catching, and in tying of the urinary papilla, were not followed by an initial period of anuria. The urinary chloride concentration varies from 0 to 87 millimols per liter, with values from 0 to 10 millimols in 16 animals. From a consideration of Fig. 2, it must be concluded that in the flounder, as in the sculpin, there is no direct relationship between the rate of urine flow and the urinary chloride concentration.

Orthagoriscus mola

Advantage was taken of an opportunity to obtain specimens of the relatively rare *Orthagoriscus mola* from a large tunny-net in the Bay of Naples. The fish were removed from the net as soon as it was sufficiently close-hauled, and urine was obtained at once by syringe from the exposed bladder. In 33 specimens the urinary chloride concentration varied from 20 to 137 millimols per liter, with an even distribution over the entire range. Although, despite many attempts, we were unable to obtain a single specimen showing a chloride-free urine, the relatively low values obtained in some cases indicate the ability of this species to excrete such a urine. The question at issue is whether the presence of chloride in the urine of these fish may be attributed entirely to skin injury or other factors associated with their presence in the net. It is our belief that this is not the case, and that the urinary excretion of chloride in variable amounts is probably a normal, physiological process in *Orthagoriscus*, for the reasons given in the footnote.[4]

Scorpæna scrofa

Urine was obtained from nine specimens of *Scorpæna scrofa* which were thoroughly acclimatized to the life in the aquarium at the Stazione Zoologica in Naples. The chloride concentrations were 0, 6, 118, 143, 145, 148, 149, 155, and 163 millimols per liter. Urine was likewise removed from five specimens which had become entangled in a net (rete di posta, Stellnetz) which had been in the water eight hours before hauling. Here the chloride concentrations were 5, 36, 107, 107, and 144 millimols per liter. These data demonstrate at once the ability of the kidney of this species to elaborate a chloride-free urine. When examined in conjunction with the plasma chloride values obtained upon such specimens, the aquarium data furthermore indicate that urinary chloride excretion may well be a physiological process in

[4] The net was of tremendous size, and had been in the water for months. *Orthagoriscus* is a very sluggish swimmer, swam lazily about in the net with no evidence of excitement, and on no day when specimens were obtained was any other species present in the net to disturb them. The net was hauled quite rapidly, and only in the last short period of close-hauling were the fishes crowded and in contact. The bladder capacity is very large, and in the specimens taken for study the urine volume was likewise very large. Under the circumstances, it seems not unreasonable to assume that the fish were under normal conditions up to the time of hauling the net. Granting this assumption, and taking into consideration our knowledge of the variations in urine flow and chloride excretion under experimental conditions in other marine teleosts, it is inconceivable that the urine of the majority of these fish could have been chloride-free up to the last short period of hauling. Those showing low urinary chloride concentration, on the other hand, might conceivably have been chloride-free.

Scorpæna. Thus, 6 aquarium specimens showed a plasma chloride concentration varying from 147.6 to 157.9 millimols per liter, with an average of 151.3 millimols. This mean value is close to the lowest average (150.6 millimols per liter—*Gadus pollachius*) obtained in a rather extensive study of various marine teleosts bled immediately after being caught with hook and line (Grafflin, 1935). On the basis of our present knowledge, this observation apparently justifies the conclusion that the aquarium specimens of *Scorpæna* are to be considered as essentially normal. Three of these specimens, with plasma chloride concentrations of 149.6, 150.8, and 151.2 millimols per liter, gave simultaneous urinary chloride concentrations of 148, 143, and 149 millimols per liter respectively.

Miscellaneous Fishes

Single specimens of *Trigla corax* (Naples aquarium) and *Gadus morrhua* (long line—Plymouth) gave urinary chloride concentrations of 8 and 5 millimols per liter respectively, and demonstrate the ability of these two species to excrete an essentially chloride-free urine. Four specimens taken at Plymouth in a steam trawl which had been dragged for about an hour yielded the following results: *Lophius piscatorius*, 132 and 139 millimols per liter; *Zeus faber*, 35 millimols per liter; *Pleuronectes platessa*, 151 millimols per liter. The urine of these fish had a marked yellow color and the quantity was rather large. The steam trawl is admittedly a rough instrument; but under the circumstances it is inconceivable that the urine of the specimens of *Lophius* and *P. platessa* could have been chloride-free before catching. In the case of *Zeus faber* such a possibility could be more readily considered. In regard to *Lophius* the following data are of interest. Pitts (1934) has reported analyses on three specimens taken in an otter trawl, with urinary chloride concentrations of 2, 6, and 98 millimols per liter. Grollman (1929) has reported a chloride analysis of 56 millimols per liter on a pooled specimen of urine collected from many goosefish taken by steam trawl in the open ocean (George's Banks fishing grounds in the Atlantic). The logical interpretation of his figure is that some of the fish showed a lower and some a higher urinary chloride concentration. When the present data are taken in conjunction with those of Pitts and Grollman, it seems not unreasonable to conclude that, while the goosefish obviously has the ability to secrete a chloride-free urine, it probably can, and not infrequently does, excrete variable, and often considerable, amounts of chloride in the urine under normal conditions.

Discussion

The present observations upon urine flow and urinary chloride in marine teleosts have led to the following conclusions: (1) that freshly caught sculpins and flounders, under fairly ideal and constant experimental conditions, show a rather wide variation in the rate of urine flow and the urinary chloride concentration; (2) that there is no direct relationship between the rate of urine flow and the urinary chloride concentration. When examined in conjunction with previously recorded observations, particularly those of Pitts (1934), the present data apparently justify a third conclusion: (3) that while all marine teleosts apparently have the *capacity* to excrete a chloride-free urine, and in their normal habitat do so in the majority of instances, they not infrequently excrete variable, and at times considerable, amounts of chloride in the urine under normal physiological conditions. In addition to his data upon sculpin and goosefish, which have been discussed above and which bear out the above conclusion, Pitts presents other data upon grey sole, hake and rusty dab which lend additional support to this thesis. Obviously, the ideal material for the final solution of the problem is original bladder urine removed from large numbers of fishes of different species immediately after catching on hook and line. However, such material is exceedingly difficult to obtain. Pitts' statement that "the vast majority of fish when caught have no urine in their bladders" has been abundantly confirmed in our own experience. With respect to his data, Pitts suggests that "the relatively high per cent of fish (eleven out of a total of nineteen) [5] reported in Table I having chloride in their urine may be a result of selection for fish which, due to some condition of the normal environment, are diuretic"; and further states that "the chance of obtaining sufficient urine for analysis from a fish increases as it becomes more diuretic." *A priori*, there seems to be little basis for believing that a diuretic fish would hold his urine on catching any more consistently than a non-diuretic fish. Furthermore, the relatively large number of Pitts' freshly caught sculpins (6 out of 15) showing considerable amounts of chloride (34 to 147 millimols per liter) in their original bladder urine can hardly be accepted as diuretic (abnormal) without further proof, particularly in view of the demonstrated independence of the rate of urine flow and the urinary chloride concentration. Pitts was led to the tentative conclusion that "marine teleosts, in general, have normally a low or zero urinary chloride." All things considered,

[5] Actually nine out of nineteen, since two of the goosefish are essentially chloride-free (2 and 6 millimols per liter).

it is our belief that the alternative conclusion is more acceptable; namely, that marine teleosts normally excrete an essentially chloride-free urine in the majority of instances, but not infrequently excrete variable, and at times considerable, amounts of chloride in the urine under normal physiological conditions.

In keeping with this latter thesis is Grafflin and Ennis's (1934) interpretation of urinary chloride as incidental to the excretion of other salts. A further implication concerns the question of a renal threshold for chloride in the marine teleosts. Marshall (1930) states: "That such a threshold for chloride exists in the case of the glomerular fish kidney is shown by examining urine from eels in salt and fresh water. In the former case the urine may contain large amounts of chloride, in the latter it rarely contains more than a trace." Yet, in the light of our present knowledge, eels in sea water almost certainly excrete an essentially chloride-free urine in the majority of instances. Furthermore, Marshall's data in the aglomerular toadfish demonstrate a a marked independence of the plasma and urinary chloride concentrations. The conclusion is drawn that the presence of a renal threshold for chloride in the marine teleosts has not been proved, and that such a threshold probably does not exist.

The situation is obviously quite complicated in the marine teleosts. We have as yet no way of determining accurately the normal rate of urine flow under conditions of existence in the sea; and there is excellent evidence that the greater part of the chloride absorbed from the gastro-intestinal tract is excreted by some extrarenal route, presumably the gills (Smith, 1930, 1932). Data obtained under fairly ideal experimental conditions have demonstrated a rather wide variation in urine flow and urinary chloride concentration, with no direct relationship between them. Furthermore, there is ample evidence to show that the presence of chloride in the urine cannot be accepted as a criterion of abnormality without further proof. From this experimental basis, the deliberate selection of animals with a low rate of urine flow (4 to 5 cc. per kilogram per 24 hours, and below) and a low or zero urinary chloride as representing the normal (see Clarke, 1934) would have to be considered as rather artificial. On the other hand, in view of the complexity of the factors involved, such a selection is undoubtedly the safest procedure to follow in physiological work until more adequate information is available.[6]

[6] When examined in conjunction with the present data upon urine flow and urinary chloride in the sculpin, the observations of Clarke (1934) upon xylose clearance in the same species are particularly interesting. His data indicate a rather constant mechanism of urine formation, in terms of glomerular filtration and tubular reabsorption of water (xylose U/P ratio = 4), up to a urine flow of about 10 cc. per kilogram per 24 hours.

The present study has shown that incomplete emptying of the bladder at the beginning of the urine collection period is a potential source of serious error in experimental work upon the fishes; and it is suggested that catheterization, rather than pressure alone, should be used in the preliminary emptying, and that relatively long collection periods should be used when it is practicable to do so.

The diuresis which develops in marine teleosts under the usual experimental conditions of handling has recently been seriously misinterpreted by Defrise (1934). In discussing our initial investigation of this problem (Grafflin, 1931), he speaks of the diuresis as being associated with a "continuous and progressively increasing cutaneous absorption of chloride and magnesium" (p. 697). Such a mechanism was not even implied,[7] and in subsequent writings (Smith, 1932; Pitts, 1934; Grafflin and Ennis, 1934; Clarke, 1934) there is general agreement that the salts enter the body only through the ingestion and gastro-intestinal absorption of sea water.

Summary

The present observations upon urine flow and urinary chloride in marine teleosts have led to the following conclusions: (1) that freshly caught sculpins and flounders, under fairly ideal and constant experimental conditions, show a rather wide variation in the rate of urine flow and the urinary chloride concentration; (2) that there is no direct relationship between the rate of urine flow and the urinary chloride concentration. When examined in conjunction with previously recorded observations, particularly those of Pitts (1934), the present data apparently justify a third conclusion: (3) that while all marine teleosts apparently have the *capacity* to excrete a chloride-free urine, and in their normal habitat do so in the majority of instances, they not infrequently excrete variable, and at times considerable, amounts of chloride in the urine under normal physiological conditions.

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[7] ". . . it seems reasonable to assume that the diuresis occurring in sculpin and toadfish with handling is dependent upon increased swallowing of sea water, brought about in some manner by the skin injury" (Grafflin, 1931, p. 609).

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THE FORMATION OF GIANT POLAR BODIES IN CENTRIFUGED EGGS OF ILYANASSA

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Introduction

The egg of the marine snail *Ilyanassa* produces a polar lobe at each of the maturation divisions and the first two cleavages. The polar lobe is normally filled with yolk and at the first cleavage passes into only one of the first two blastomeres and is thus responsible for the unequal first cleavage. Morgan (1933), in a study of the factors involved in the formation of the polar lobe, found that if eggs of *Ilyanassa* are held upside down on the centrifuge so that the normal stratification of yolk and cytoplasm is reversed, the polar lobe continues to form irrespective of the visible oöplasmic content of the region and irrespective of the position of the mitotic figure. It was observed also that if the third lobe (formed at the first cleavage) is detached from the egg, it continues to undergo changes in shape. From these results Morgan was led to infer that lobe formation ". . . Is to some extent an autonomous phenomenon, the result of conditions in the antipolar surface of the egg which is not affected by the centrifuging of the materials in its interior." In a recent series of papers Morgan (1935*a*, *b*, *c*) has extended these observations and has reported the behavior of fragments of eggs separated by centrifugal force. He finds that fragments from the animal hemisphere which contain both egg and sperm nuclei develop without any indication of lobe formation, whereas non-nucleated fragments from the vegetal hemisphere undergo rhythmic changes in shape to produce polar lobes. Unlike the normal egg, the fragments from the animal hemisphere divide into equal cells at the first and second cleavages, while at the third cleavage four micromeres are formed in normal fashion. During the summer of 1934 the author performed independently at Woods Hole a similar series of observations on animal and vegetal halves of the egg of *Ilyanassa* separated by centrifugal force. The results were essentially similar to those obtained by Morgan. Fragments consisting of the animal half of the egg were observed to develop for two or three days, giving rise to ciliated embryos capable of some slight degree of locomotion.

These observations show that the behavior of isolated animal and vegetal parts of the egg of *Ilyanassa* is, with respect to lobe formation, similar to that of corresponding parts of the egg of *Dentalium*, as described many years ago by Wilson (1904). In *Dentalium* the capacity to produce the polar lobe is restricted even before fertilization to the lower half of the egg. And if after fertilization the lower half is isolated without a nucleus, it continues to form the polar lobe as if it were still a part of the whole egg. The autonomous nature of the lobe-forming process is especially interesting in view of the important rôle which its materials play in determining development (see especially Wilson, 1904, on *Dentalium*, and Crampton, 1896, on *Ilyanassa*).

In his recent paper Morgan (1935*b*) mentions the possibility that occasional protoplasmic protrusions which he has observed in centrifuged eggs of *Ilyanassa* may be giant polar bodies, similar to those described by Conklin (1917) in centrifuged eggs of *Crepidula*. He is somewhat skeptical, however, of the validity of this interpretation. In the present paper evidence will be presented to show that giant polar bodies do form in centrifuged eggs of *Ilyanassa* and that occasionally they, instead of what would ordinarily be considered the egg, undergo development. In addition, the rather unusual stratification of egg substances under strong centrifugal force is described. Part of these observations were made at Woods Hole and part were made on eggs from snails maintained in the laboratory at Princeton. The author wishes to thank Professor E. G. Conklin for his interest in this work, and for helpful advice and criticisms concerning it.

Giant Polar Bodies

When eggs of *Ilyanassa* are suspended in a medium of equal density and centrifuged, they orient with their yolk-laden vegetal poles outward. The egg substances are easily stratified into three zones: oil at the animal pole, yolk at the vegetal pole, and granular cytoplasm in between. Nuclei come to lie in the cytoplasmic zone. Maturation spindles, at and after the metaphase stage, are anchored to the periphery at the animal pole and hence offer resistance to displacement. If, however, the eggs are so oriented on the centrifuge that the point of attachment of the maturation spindle lies well within the region where the oil cap is formed, the incrowding of the oil droplets may force the denser spindle away from its point of attachment. The spindle may thus come to lie wholly or partly in the cytoplasmic zone. In such cases, strands of denser cytoplasm may be seen, in fixed and stained preparations, connecting one end of the maturation spindle with the periphery at the animal pole (Fig. 14). The cell wall at the point of

attachment of these strands may be indented by the pull exerted on it during centrifuging.

Whether normal or abnormal polar bodies form in such centrifuged eggs depends on the position of the maturation spindle when the division takes place. If the spindle returns to its original position at the animal pole, a normal polar body may be formed. If it does not return before division sets in a giant polar body is formed. Smaller "giants" may contain only a part of the oil cap (Fig. 18), larger ones may contain all of the oil cap (Fig. 16), and still larger ones may contain a part of the cytoplasmic zone (Fig. 17). In experiments to be described in this paper the eggs were centrifuged after the formation of the first polar body and before the formation of the second. The position of the first polar body thus served as a convenient marker of the animal pole. When giant polar bodies were formed they resulted from the second maturation division. The fate of such giant polar bodies varied. In some cases the giant polar body underwent no further development, while the egg proper developed normally. In other cases the egg proper did not cleave, although it did undergo rhythmic contractions to form the polar lobe, while the giant polar body underwent development. Whether the giant polar body or the egg proper is to develop seems, without doubt, to depend on the presence or absence of the sperm nucleus. In eggs centrifuged in the interval between the maturation divisions, or earlier, the condensed sperm nucleus comes to lie in the cytoplasmic zone, near the boundary between the latter and the oil zone (Figs. 14, 15, 23). In cases where the giant polar body includes a portion of the cytoplasmic zone, it would sometimes include also the sperm nucleus. The giant polar body would then be fertilized by the sperm nucleus and would therefore develop, while the part that would ordinarily be considered the egg, although containing the female pronucleus, would be unfertilized and hence would not develop.

Experiments

The data from three experiments will be summarised to illustrate the production of giant polar bodies. In all cases the eggs were suspended in a gum arabic-sea water solution and rotated on an electric centrifuge equipped with hæmatocrit head.

1. Beginning 17 minutes before the second polar body was due, eggs were centrifuged at a moderate speed for 7 minutes. Three zones were separated and the eggs slightly elongated (Fig. 1). Thirty minutes after the eggs were removed from the centrifuge a constriction between the cytoplasmic and oil zones announced the beginning of the

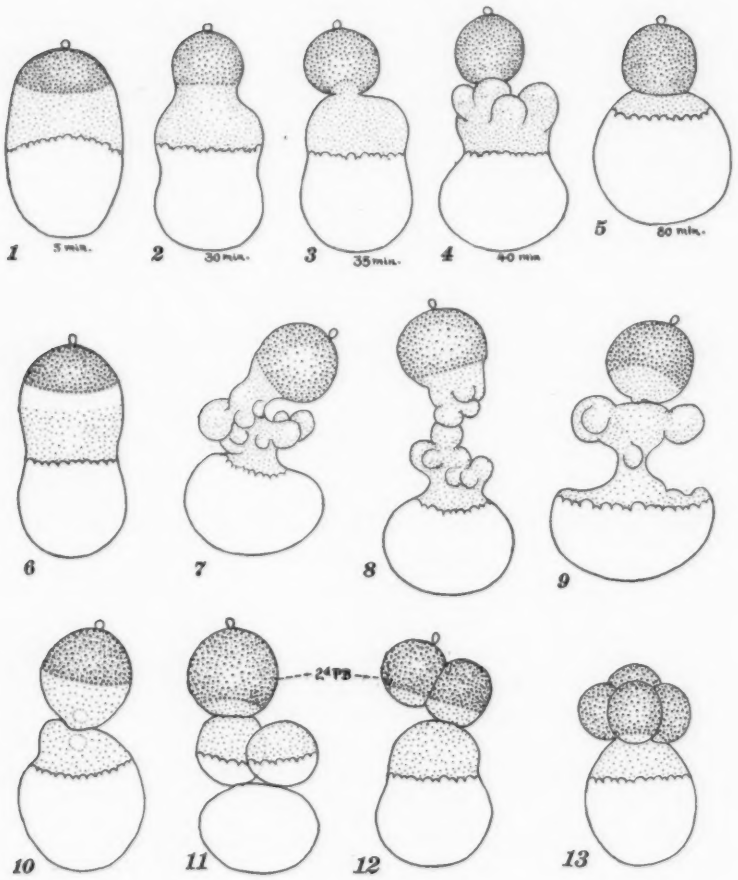

PLATE I

FIGS. 1–5. Same egg at successive intervals after centrifuging, showing formation of giant second polar body. See Experiment 1 of text. 1, 5 minutes; 2, 30 minutes; 3, 35 minutes; 4, 40 minutes; 5, 80 minutes.

FIGS. 6–13. Showing behavior of eggs centrifuged in interval between maturation divisions. See Experiment 2 of text.

6. Egg 5 minutes after centrifuging.

7–9. Three different eggs 40–45 minutes after centrifuging. Second maturation division producing giant polar bodies. Note cytoplasmic lobes.

10. Egg 70 minutes after centrifuging; giant second polar body.

11. Egg 2½ hrs. after centrifuging. Trefoil stage of first cleavage; polar body undivided.

12. Another egg 2½ hrs. after centrifuging. Egg undivided; giant polar body cleaved.

13. Egg 3½ hrs. after centrifuging. Giant polar body in 4-cell stage.

second maturation division; a constriction in the yolk zone represented formation of the polar lobe (Fig. 2). Ten minutes later the oil zone was constricted off as a giant second polar body and around the animal end of the egg a cluster of cytoplasmic lobes had appeared (Fig. 4). Six minutes later the cytoplasmic lobes had been resorbed. The polar lobe constriction persisted for some minutes longer, but eventually disappeared (Fig. 5). An hour after the giant polar bodies were formed a part of the eggs were fixed and stained. Two of them are shown in Figs. 16 and 17. The first polar body is normal, the second is large and contains the oil cap (Fig. 16) or oil cap plus part of the cytoplasmic zone (Fig. 17), as well as chromatin from the second maturation division. Two hours and forty minutes after the giant second polar bodies were formed the first cleavage had occurred; the eggs were fixed and stained. In some cases the egg showed normal first cleavage (Fig. 19); the nuclear membrane in the giant polar body had dissolved and the chromatin was lying free in the cytoplasm. In other cases the egg proper showed a polar lobe constriction but no cleavage, whereas the giant second polar body had cleaved (Fig. 20). The two small cells resulting from the division of the giant polar body contained normal looking nuclei; the egg proper contained chromosomal vesicles from the second maturation division.

2. Beginning 12 minutes before the second polar body was due another lot of eggs was subjected at alternate 5-minute intervals to centrifuging and rest over a 30-minute period (Fig. 6). Forty minutes after the eggs were removed from the centrifuge and one hour after the second polar body was given off in the controls, the second maturation division occurred. The division plane cut through the cytoplasmic zone, thus giving rise to giant polar bodies. At the time when the division was just being completed, the cytoplasm of both giant polar body and egg proper protruded numerous lobes. These persisted for several minutes and were then resorbed (Figs. 7–10). One hour and forty minutes after the giant second polar body was formed, the first cleavage had occurred. In some cases the egg proper cleaved and the giant polar body underwent no change (Fig. 11), while in other cases the egg proper remained uncleaved and the giant polar body cleaved (Fig. 12). One hour later the giant polar body was in the four-cell stage (Fig. 13); a fixed and stained preparation is shown in Fig. 21.

3. In this experiment the eggs were broken into animal and vegetal halves. Eggs with the first polar body off were centrifuged slowly for 25 minutes (11:13–11:38 A.M.); they were greatly drawn out in the middle so that only long, slender necks of protoplasm connected the animal and vegetal halves. By agitation with a stream of water from

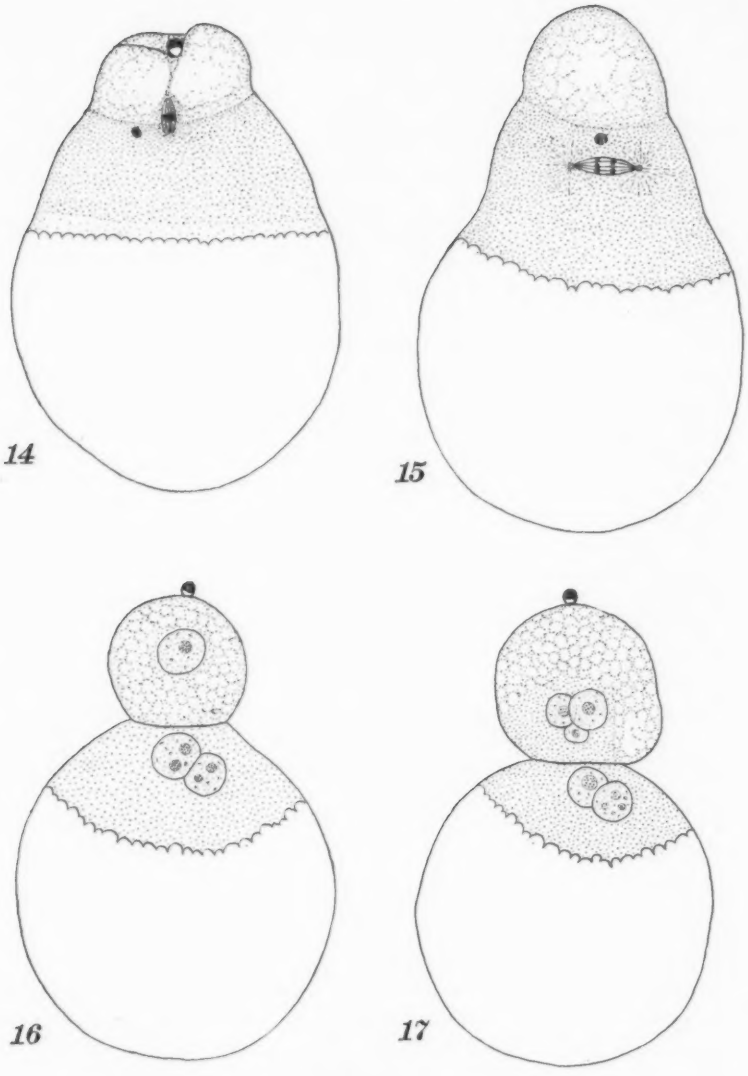

PLATE II

FIG. 14. Egg centrifuged for 5 minutes. Second maturation spindle forced away from animal pole but remains connected by strands of cytoplasm. Condensed sperm nucleus at border of oil and cytoplasmic zones.

FIG. 15. Egg centrifuged intermittently over 30-minute interval (See Experiment 2 of text), then fixed immediately. First polar body lost. Second maturation spindle lying free in cytoplasmic zone. Sperm nucleus between spindle and oil zone.

FIGS. 16, 17. Giant polar bodies in eggs centrifuged before second maturation division (See Experiment 1 of text).

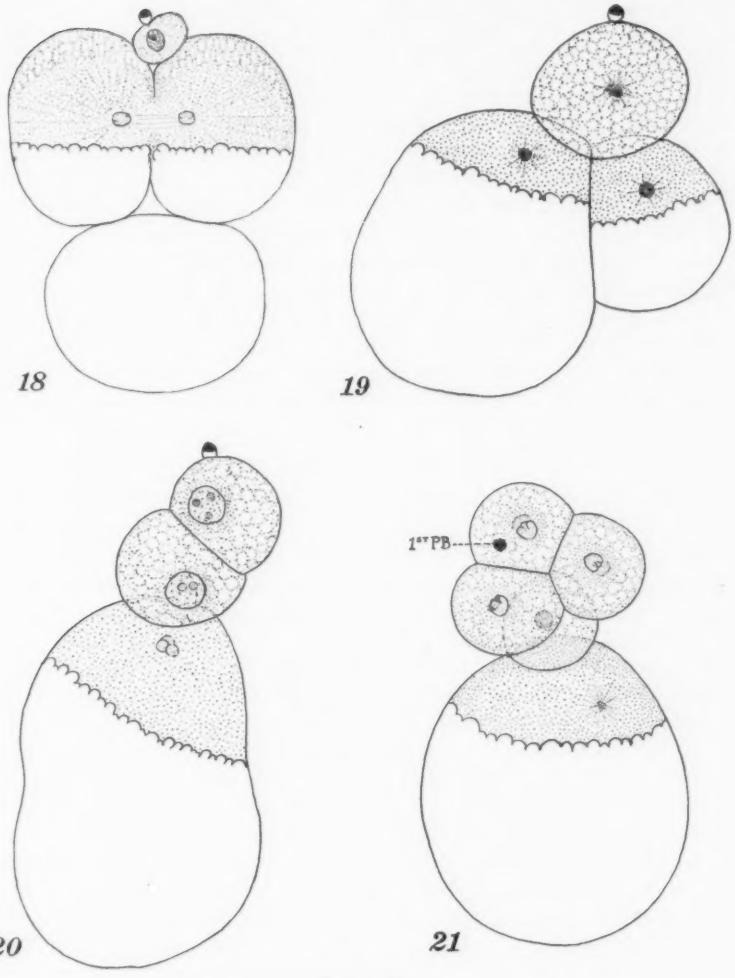

PLATE III

Eggs from different lots centrifuged in interval between maturation divisions and preserved later.

FIG. 18. Second polar body larger than normal; egg in trefoil stage of first cleavage.

FIG. 19. Giant second polar body contains all of oil zone. First cleavage of egg normal.

FIG. 20. Giant second polar body has divided; egg undivided.

FIG. 21. Egg undivided, giant second polar body in 4-cell stage.

a fine pipette the animal and vegetal halves were separated. At 12:15 P.M. the vegetal halves showed polar lobe constrictions and the animal halves were beginning to divide. By 12:20 the animal halves had divided equatorially into two equal cells (second maturation division); both parts showed, during the succeeding minutes, small cytoplasmic lobes, as described in the preceding experiments. At 12:30 the two cells of the animal half were round, and likewise the isolated vegetal halves were round. At 1:25 the vegetal halves were again constricted, and the polar moiety (giant polar body) of the animal halves cleaved into two cells. The vegetal halves now became spherical again, but at 3:30 showed another constriction; at this time the giant polar body underwent a second cleavage. Obviously, in this experiment the sperm nucleus was included in the giant second polar body; the central cytoplasmic part contained chromatin from the second maturation division, but no sperm nucleus.

The Zones of the Centrifuged Egg

With moderate centrifuging the contents of the egg of *Ilyanassa* are divided into three zones. Since the eggs orient on the centrifuge, the yolk particles are merely concentrated in the vegetal half; oil drops accumulate as a cap around the animal pole; the cytoplasmic zone which lies between these two contains many fine, colorless granules, as well as scattered oil droplets and smaller yolk particles. If eggs are subjected to harder and more prolonged centrifuging, further separation of substances takes place. First, the fine granules of the middle zone are thrown down so that a hyaline zone appears beneath the oil cap. At this stage the eggs may pull into halves. In other cases further stratification takes place. A second clear band appears in the cytoplasm just above the yolk zone. Five zones are now present: (1) oil cap, (2) upper clear zone, (3) fine, colorless granules, (4) lower clear zone, and (5) yolk (Fig. 22). This stratification has been observed in fertilized eggs of various stages and in unfertilized eggs (obtained by tearing apart the body of the female) in which the germinal vesicle was still intact. Eggs are more resistant to stratification before the germinal vesicle has dissolved than afterwards. A similar decrease in cytoplasmic viscosity upon the breakdown of the germinal vesicle has been noted by many observers in a variety of other eggs. The germinal vesicle in the centrifuged *Ilyanassa* egg comes to lie beneath the oil cap. Within the germinal vesicle chromatin and the large nucleolus are thrown to the centrifugal end.

The nature of the five zones of the fully stratified egg will now be

considered. The lightest and heaviest zones consist, respectively, of oil droplets and yolk particles. The upper clear zone consists of hyaline protoplasm which in living eggs appears homogeneous and structureless. The fine, colorless granules of the central band stain readily with Janus Green B. This is best observed by placing eggs in

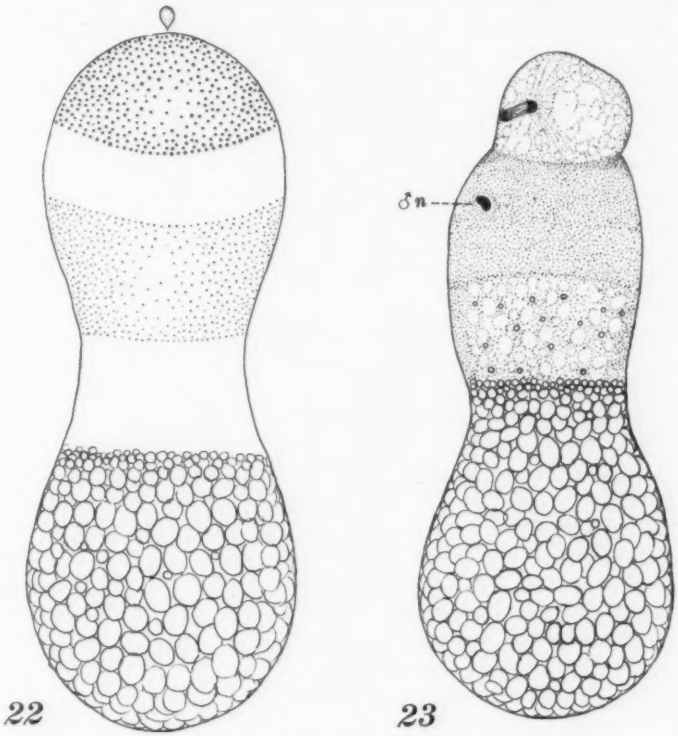

PLATE IV

FIG. 22. Living egg, centrifuged for 19 minutes. Five zones of materials separated: oil; upper clear zone; fine, colorless granules; lower clear zone; yolk.

FIG. 23. Fixed and stained preparation of whole egg, centrifuged during anaphase of first maturation division; upper clear zone granular, lower clear zone vacuolated.

a weak solution of the dye for a few minutes before centrifuging. Upon centrifuging, the colored band of granules then stands out quite clearly. The size and relative density of these granules and their affinity for Janus Green B indicate that they are mitochondria.

However, in sections of centrifuged eggs prepared according to the Altmann technic for mitochondria, these granules showed no selective staining, but the yolk particles instead took up acid fuchsin. In spite of this discrepancy, it seems likely that they are comparable to the fine, colorless granules of other eggs, notably those of the sea urchin egg (Wilson, 1926; Harvey, 1933), which have been identified as mitochondria. The lower or heavier clear zone is quite different from the upper one. In centrifuged eggs which had been killed in formalin, treated with Sudan III and cleared in glycerin, the upper clear zone appeared finely granular, while the lower clear zone showed a meshwork of faintly visible cytoplasmic strands amongst which could be seen lacunæ or alveoli and a few small yolk spherules. In centrifuged eggs fixed in Kleinenberg's picro-sulfuric fluid and stained in Conklin's modification of Delafield's hæmatoxylin, the upper zone stains freely and appears densely granular, whereas the lower zone stains faintly, is not so granular, and has a faintly but definitely vacuolated appearance (Fig. 23).

Such a stratification as this may mean either (1) that under centrifugal force a gradient of density is established in the clear cytoplasm and that the fine granules possess a specific gravity intermediate between that of the heavier and lighter cytoplasms, or (2) that the fine granules are thrown down against a preformed region of gelated or partially gelated hyaline protoplasm and that they pile up against this zone to form a band. The latter interpretation seems to be the more likely one, especially in view of the difficulty of displacing yolk particles from this region.

Discussion

The formation of giant polar bodies in centrifuged eggs of *Ilyanassa*, as described in this paper, is essentially similar to that observed by Conklin (1917) in eggs of *Crepidula* centrifuged during the maturation period. For greater cytological details and a comprehensive review of the subject, the reader is referred to his paper. Centrifuging *Crepidula* eggs in sea water, Conklin found that by mutual pressure the eggs were prevented from orienting in the tubes so that in many cases yolk was thrown to the animal pole. Thus maturation spindles could be forced far away from their normal positions. Polar bodies of varying size and composition could be given off at any position on the egg surface, even at the vegetal pole. Displaced nuclei and cytoplasm in time returned to the animal pole, however, so that the polarity of the egg remained unchanged. Giant polar bodies did not develop because they did not receive a spermatozoön and they did not receive a spermatozoön because they formed after the egg had been

fertilized and the cortical layer rendered impervious to further sperm entry. In some cases, however, the daughter cell of the second maturation division which lay at the animal pole and which in this paper has been referred to as the giant polar body, received the sperm nucleus and hence developed. That part of the egg which developed Conklin referred to as the egg, whereas the other part, whether at the animal or vegetal pole, he referred to as the giant polar body.

The peculiar activity of the egg which results in the production of clusters of cytoplasmic lobes at the close of the second maturation division is reminiscent of similar cytoplasmic activity in dividing tissue-culture cells, as demonstrated by Canti and by the Lewises in motion-pictures. Somewhat similar lobe production at the time of the maturation divisions has also been observed by Morgan and Tyler (1935) in centrifuged eggs of *Urechis*. The normal egg of *Ilyanassa*, it will be remembered, produces a polar lobe at the time of the maturation divisions. The cluster of cytoplasmic lobes around the region of the fading maturation figure in centrifuged eggs is another phenomenon, however, as is evident from the behavior of isolated animal and vegetal halves of the egg: the isolated vegetal half continues to form the polar lobe at the usual times, while the nucleated animal half does not form the polar lobe, but does produce the cluster of cytoplasmic lobes. These cytoplasmic lobes evidently result from some change in the egg accompanying the division process. They have not, however, been observed in the normal egg. It may be that they appear in the centrifuged and elongated egg because the cell wall and cortex have been weakened by undue stretching.

The usual stratification of substances in strongly centrifuged eggs includes a layer of oil droplets at the centripetal pole, two or three layers of granules (mitochondria, yolk, pigment, etc.) at the centrifugal pole, and an intermediate zone of hyaline protoplasm. Some exceptions are known. In *Tubifex*, according to Parseval (1922), the yolk occupies an intermediate position between two clear zones. In *Sphærechinus granularis*, Ethel B. Harvey (1933) has observed two zones of clear protoplasm, one in the usual position next to the oil cap and the other at the centrifugal pole. *Ilyanassa* differs from the usual situation in that two clear layers are present and separated by a band of fine granules. As has been pointed out, this evidently means either (1) that a gradient of density is established in the clear cytoplasm, or (2) that the more centrifugal clear zone represents a region so firm in consistency that the fine granules cannot penetrate it. In this latter respect it may resemble the central spongy area of the egg of *Chætopterus*, as described by Lillie (1909), which by virtue of its resistant wall

neither allows granules from other regions to pass through it nor allows those granules within it to pass out of it, except with very strong centrifugal force.

Summary

1. If the second maturation spindle of the egg of *Ilyanassa* is displaced from the animal pole by centrifugal force, a giant polar body may be formed. If the sperm nucleus is included in the giant polar body the latter, instead of the egg proper, may develop.

2. The close of the maturation division in centrifuged and elongated eggs is attended by the production of clusters of cytoplasmic lobes around the region where the achromatic figure lay. These lobes are distinct from the normal polar lobe.

3. In strongly centrifuged eggs five zones of substances are separated. These include two clear zones separated by a band of fine, colorless granules.

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THE EFFECT OF TEMPERATURE ON THE RATE OF THE FERTILIZATION REACTION IN VARIOUS MARINE OVA

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INTRODUCTION

The development of a fertilization membrane in most marine ova serves as an indicator of the fertilization reaction. The process is important because of its relation to the cortical reaction of the egg and its conspicuous part in it. Loeb (1906, 1910, 1916) and Loeb and Wasteneys (1915) have presented a large body of evidence supporting the view that the essential feature of fertilization is the increase in the rate of oxidations and that this increase is caused by membrane formation, or more strictly, by the reactions normally resulting in the development of the fertilization membrane. More recently Chase (1935) has shown that the fertilization membrane develops in some eggs by a radical transformation of the vitelline membrane, in others chiefly by the separation of a tough pre-existing membrane from the surface of the egg. Loeb's work was done mainly on the sea urchin egg, which belongs in the former category. It is not improbable that the development of the fertilization membrane in part may be a different type of process in eggs which have a tough membrane before activation.

This paper reports a study of the effect of temperature on the development of the fertilization membrane in several kinds of eggs. Measurements on the effect of temperature fall in the following categories: (*A*) effect on gametes of exposure to various temperatures, (*B*) the effect of temperature on the time until contact of the sperm with the egg, and (*C*) the effect of temperature on the rate of the development of the fertilization membrane.

MATERIAL AND METHODS

The experiments were performed during the spring and summer of 1933 and the winter and spring of 1934 at the Hopkins Marine Station, Pacific Grove, California.[2] Eggs of the following species were used:

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the sand dollar, *Dendraster excentricus;* the sea urchin, *Strongylocentrotus purpuratus;* the starfish, *Patiria (Asterina) miniata;* and the echiuroid, *Urechis caupo,* Fisher and MacGinitie (1928*a*, 1928*b*), These forms are found in abundance in the vicinity of the station.

Animals (except *Urechis*) were collected each day when an experiment was made in order to insure that mature gametes in the best condition were used. Sand dollars were dredged from Monterey Bay and sea urchins and starfish were collected at low tide from pools where the water was fairly deep. The large worm, *Urechis,* which lives in U-shaped burrows in mud flats of estuaries along the California Coast, was dug at Elkhorn Slough, about 20 miles from the station, when the tides were favorable.

Gametes of the echinids were obtained by cutting out the gonads of the animals. Bits of ovary were placed in finger bowls containing sea water and removed after the eggs had exuded. Portions of testis were placed in clean dry Syracuse watch glasses where the sperm were shed. Starfish were induced to spawn when they were brought into the laboratory and spread on wet sea weed, and the gametes were pipetted off as they were extruded. The eggs were placed in finger bowls containing sea water and the sperm were placed in Syracuse watch glasses. Eggs and sperm of *Urechis* were collected by means of a small bore pipette inserted into the gonopore of an animal. The eggs were kept in finger bowls containing sea water and the sperm were placed in Syracuse watch glasses.

Standard sperm suspensions were made as they were needed by adding a drop of dry sperm to 5 cc. of sea water. Every precaution was taken during the handling of the eggs to prevent premature activation. Samples from each finger bowl were observed frequently and when eggs with fertilization membranes were found the whole dish was rejected.

For regulation of temperature a water bath with a transparent bottom was constructed and fitted to the microscope. A flat-bottomed glass vessel, the fertilization chamber which contained sea water, was placed within the bath. A glass slide upon which the gametes were placed was suspended within this chamber so that it was below the surface of the sea water and within the focus of the microscope. The bulb of a calibrated thermometer rested on the slide adjacent to the gametes. Any desired temperature down to 2° C. could be maintained constant to within $\pm$ 0.25° C.

All observations of eggs and sperm were made at a magnification of 180 diameters.

The Experiments

Since it is generally known that the development of many marine eggs is slower at low temperatures than at higher temperatures, it is important to know to what extent changes in temperature affect those initial reactions which underlie the developmental process. The present section details experimental results concerning the effect of temperature on the rate of the cortical reaction.

Effect on Gametes of Exposure to Various Temperatures

Gametes were subjected to a range of temperature from 2° C. to the highest viable temperatures. Observations were made on the activity of sperm at extreme temperatures as compared with the activity of sperm in a control run at 15° C. (the approximate average temperature of water in Monterey Bay). After temperature adjustment a drop of fresh sperm suspension was placed in the fertilization chamber and observed microscopically. At 2° C. sperm of *Dendraster* and *Patiria* became feebly active while sperm of *Strongylocentrotus* and *Urechis* were very active. At temperatures of 4° C.–10° C. sperm of all species showed increased activity with rise in temperature. When sperm of each species were observed at 25° C. they were found to be very active but at 28.5° C.–30° C. sperm of *Dendraster* and *Patiria* became inactive. The sperm of *Strongylocentrotus* and *Urechis* were slightly more resistant to the higher temperatures. They became inactive between 35° C. and 40° C.

Unfertilized eggs were observed at extreme temperatures. At 2° C. eggs of *Dendraster*, *Strongylocentrotus*, and *Urechis* were apparently not affected by cold. However, starfish eggs were activated by very low temperatures as was shown by Greeley (1902). Eggs of *Patiria* were affected by temperatures between 2° C. and 7° C. Immediately after the eggs were dropped into the sea water at 2° C.–4° C. large blisters formed in the outer layer of each egg and the cytoplasm receded from the egg membrane. This phenomenon was followed by the abnormal cleavage of the egg in some cases and in most cases by fragmentation. At slightly higher temperatures, 5° C.–7° C., smaller blisters formed and the egg membrane began to separate from the underlying cytoplasm which in a few cases gave a fairly regular cleavage, while in many other cases the egg fragmented. A few eggs were activated by temperatures as high as 8° C.–8.5° C. while others were more resistant to cold and were not activated at temperatures as low as 6° C. At 29° C. and above eggs were affected by heat. Eggs of *Dendraster* usually formed one or more large blisters and at 32° C.

they cytolyzed. Eggs of *Strongylocentrotus* formed blisters at 28° C.–32° C. and cytolyzed above 32° C. When eggs of *Patiria* were exposed to a temperature of 30° C. membrane separation began, followed by cytolysis. Eggs of *Urechis* were more resistant to heat than eggs of the other species, showing the beginning of the separation of membranes at 35° C. and cytolyzing at higher temperatures.

The Effect of Temperature on the Time until Contact of the Sperm with the Egg

A drop of adequately concentrated sperm suspension was placed in the fertilization chamber which contained sea water at a particular temperature. A small drop containing eggs was then placed in the chamber and the time of insemination was recorded by stop watch the instant that the eggs came into the sperm sea water. The time of sperm contact was recorded when the first sperm was seen at the surface of an egg. In each case a control was run at 15° C., and the time for contact at this temperature was compared with that at the other experimental temperatures. All eggs studied except those of *Urechis* were enclosed within a relatively thick layer of jelly. In the case of the latter the jelly was only about 5 to 10 microns thick.

The duration of the appreciable time-lapse depended principally upon the activity of the sperm at the various temperatures, but perhaps also to some extent upon the physical consistency of the jelly. During most of the time-lapse the sperm were swimming through the layer of jelly. This took longer at low temperatures. Numerous measurements were made, usually 10 to 20 or more with each species at each temperature. The final results were obtained by averaging the time between insemination, and the contact of the fastest swimming sperm with the egg, rather than averaging all of the measurements in each experiment. The data are given in Table I.

The Effect of Temperature on the Rate of Development of the Fertilization Membrane

The effect of temperature on the rate of the reactions leading to the development of the fertilization membrane was determined by inseminating eggs in the manner described in the preceding section at temperatures ranging from 2° C. to the highest temperature at which normal fertilization took place. No eggs were used unless 95 to 100 per cent of a sample developed fertilization membranes at 15° C.

In each experiment about ten eggs came to lie in the field of the microscope. Time was measured from the instant the eggs were dropped into the sperm suspension, until the beginning of the develop-

ment of the fertilization membrane was observed on that egg which represented the reaction of half of the eggs in the field of the microscope (usually the fifth egg to react).

The actual reaction-time (the time-lapse from contact of the spermatozoön with the egg to the beginning of the development of the fertilization membrane) was determined by subtracting the average

TABLE I

Approximate minimum time (seconds) for sperm to reach the egg surface at different temperatures.

Temperature	Dendraster	Strongylocentrotus	Patiria	Urechis
°C.				
2	41.3	9.3	—	10.3
3	39.1	7.1	—	10.0
4	34.1	5.6	30.4	9.6
5	28.9	5.1	25.2	8.9
7	23.0	4.3	23.9	6.7
10	17.8	3.3	17.3	3.7
12	12.0 *	2.9 *	14.0	2.6
15	5.3	2.6	13.4	1.6
17	5.0 *	2.2 *	11.3	1.8
20	4.5	1.9	9.9	1.0
22	7.5	1.9	9.1	0.9
25	8.0	2.1	7.9	0.9
27	8.9	2.2 *	6.9	1.8
27.5	9.2	—	—	—
28	9.2	—	6.1	—
28.5	13.4	—	6.6	—
29.5	—	—	5.9	—
30	—	2.4	—	3.1
31	—	2.5 *	—	—
31.5	—	2.6	—	—
32	—	2.7	—	3.8
35	—	—	—	3.8

* Interpolated values.

values of the time required for the sperm to pass through the egg jelly at a given temperature (Table I) from the time measured from insemination to the beginning of the development of the fertilization membrane. The data for the actual reaction-time are shown graphically in Fig. 1.

DISCUSSION

A study of the effect of temperature on the rate of the cortical reactions which lead to the development of the fertilization membrane gives objective information concerning the most important parts of the process, namely, the invisible reactions which underlie membrane

development and which presumably initiate the development of the egg. The fertilization membrane is of little developmental importance once it is fully formed; its development being merely the indicator of

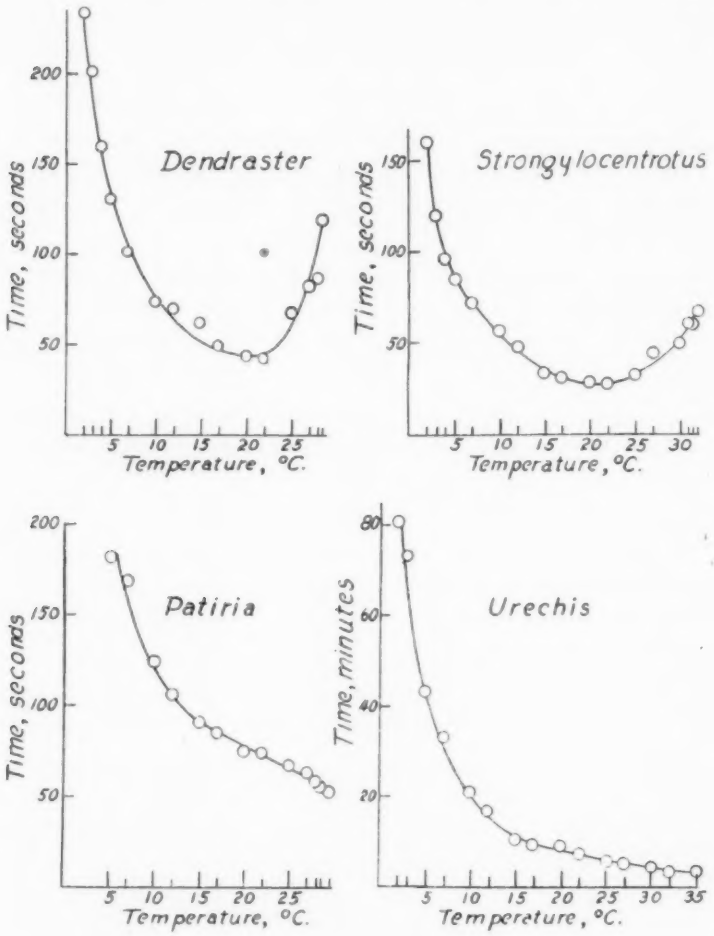

FIG. 1. Effect of temperature on the time of the beginning of the development of the fertilization membrane after contact of the sperm with the egg. Each circle represents the average of 20 to 95 measurements (average number of measurements = 43.8), each on about ten eggs.

the underlying cortical reaction. It is of interest in this connection, especially, to know when and how the development of the fertilization membrane takes place.

Explicit Definition of the Phenomenon Measured

In measuring the time-lapse from the contact of the sperm with the surface of the egg to the beginning of the development of the fertilization membrane, it must be pointed out that in none of the eggs studied does the development of the membrane begin at all points of the egg surface at the same time. It starts at one point, usually the entrance point of the sperm. After a brief time-lapse, which is greater the greater the distance from this initial point, the membrane starts to develop at all other points. Since the sperm may enter the eggs of the four species at any point, and presumably with equal probability, oftener than not the first point at which the development of the fertilization membrane begins is not in the focal plane of the microscope. In about half of the cases this point is on the under side of the egg and is not visible. The practicable locus of observation where accurate determinations can be made is the geometrical equator of the egg as seen from above, and this is the focal plane of the microscope and the place where the beginning of the development of the fertilization membrane was timed.

If the sperm entered at the equator, the observed time for the beginning of the development of the membrane would be the minimum for the whole egg. The other limit would be the entrance of the sperm at a point 90° above or below the optical equator of the egg. In this case the beginning of membrane development at the equator would be at a point one-fourth the circumference of the egg removed from the entrance point of the sperm. Since the entrance point and the resting position of the observed egg bear chance relationship, the average of the measurements would represent the time-lapse from the contact of the sperm with the egg surface to the beginning of the development of the fertilization membrane at a point one-eighth of the circumference of the egg from the entrance point of the sperm. This is the time-lapse measured in the eggs of the four species, and since each average includes hundreds of eggs, a good average has been obtained.

Temperature Coefficients

Since chemical reactions usually increase in rate with rise in temperature in such a way that the rate is approximately doubled when the temperature is increased 10° C. (Q_{10} = approximately 2.0), the order of magnitude of Q_{10} values has been used extensively in

biological work to determine whether the rate of a measurable process is limited by the rate of metabolic or chemical reaction on the one hand, or by physical factors (Snyder, 1908). Loeb and Wasteneys (1911) have shown that the Q_{10} values over the viable range for the rate of oxygen consumption by *Arbacia* eggs is approximately constant and approximately equal to 2.0. On the other hand, the temperature coefficients for the rate of cell division in this same form were shown to range from $Q_{10} = 7.3$ (7° C.–17° C.) to $Q_{10} = 1.7$ (20° C.–30° C.). The rate of cell division in the lower temperature ranges is evidently limited by factors other than metabolic rate, or as Loeb and Wasteneys conclude, oxidation is not the independent variable in development. In the middle temperature ranges, however, the temperature coefficient is that of a chemical reaction. Fauré-Fremiet (1913) finds that the Q_{10} values for cleavage rate in *Ascaris* eggs increases from -4.1 between 37° C. and 42° C. to 6.25 over the range from 6° C. to 16° C. He also concludes that factors other than chemical reactions enter into limiting the rate of cell division and he suggests viscosity as another factor, especially since he had previously shown in *Ascaris* that the viscosity had a Q_{10} value of about 5.0 to 5.8.

Ephrussi (1927) has accurately determined the temperature coefficients (Q_{10}) of various stages in mitosis in eggs of *Paracentrotus* and *Ascaris*. He found that the values agreed very closely indeed in the two forms at corresponding stages. The coefficients determined in the middle ranges of the viable temperature ranges for the forms used (18.5° C.–26° C. for *Paracentrotus;* 24° C.–34° C. for *Ascaris*) indicate that the process is chiefly physical. For example, the change in the state of nuclear material after the end of the anaphases in *Paracentrotus* shows a coefficient of $Q_{10} = 1.0$. For swelling or imbibition of the sperm nucleus $Q_{10} = 1.6$.

Snyder (1911) points out that in chemical reactions Q_{10} is not strictly constant. Bayliss (1924, p. 42) emphasizes the variability of temperature coefficients in various chemical systems and over different temperature ranges. It must be kept in mind then that temperature coefficients may be used only as a somewhat rough indication of reaction type, and that it is the order of magnitude of the Q_{10} values, rather than very exact figures, which is significant. For the comparatively simple purpose of the treatment to follow, the Van't Hoff coefficient (Q_{10}) is used rather than the temperature characteristic (μ) of the Arrhenius equation, which has been used especially by Crozier (1924) for more refined analysis of temperature effects.

The Temperature Coefficients of the Reactions Leading to the Development of the Fertilization Membrane.—Temperature coefficients (Q_{10}) were calculated from the data in Fig. 1 after Snyder (1908) as follows:

$$Q_{10} = \left(\frac{K_2}{K_1}\right)\frac{10}{t_1 - t_2},$$

where K_2 is the time-lapse at the lower temperature t_2 and K_1 is the time-lapse at the higher temperature t_1. When the reaction rate diminishes with rise in temperature the fraction, K_2/K_1, is inverted and a negative sign is given the coefficient.

The calculated values of Q_{10} for eggs of the four species studied are given in Table II. An inspection of the table shows at once that the

TABLE II

Temperature Coefficients (Q_{10}) for Different Ranges of Temperature

Temperature interval	Q_{10} values			
	Dendraster	Strongylocentrotus	Patiria	Urechis
°C.				
2°– 4°	6.7	12.4	—	—
2°– 5°	7.0	8.1	—	8.2
2°– 7°	5.4	5.0	—	6.0
5°–10°	3.1	2.3	2.1	4.3
7°–12°	2.1	2.2	2.5	3.9
10°–15°	1.5	2.7	1.8	3.9
12°–17°	2.1	2.5	1.6	3.1
15°–20°	2.0	1.4	1.5	1.5
17°–22°	1.3	1.3	1.3	1.6
20°–25°	− 2.3	− 1.3	1.2	2.2
22°–27°	− 3.7	− 2.7	1.4	2.1
25°–28°	− 2.4	—	1.7	—
25°–30°	—	− 2.4	—	2.3
27°–32°	—	− 2.3	—	2.8
30°–32°	—	− 4.7	—	—
30°–35°	—	—	—	1.7

temperature coefficients are not constant throughout the temperature ranges of fertilization nor are they of the order of magnitude characteristic of chemical reactions except in the middle temperature ranges. As temperature decreases there is an increase from values of the order of 1.3 or 1.5 to values altogether above the range characteristic of metabolic processes, except in *Patiria* where the values do not increase so greatly. However, this exception in the case of *Patiria* may be due in part to the fact that measurements were not made at the lower temperatures, and could not be, because as previously described, cold

alone induces a sort of cytoplasmic withdrawal from regions of the egg surface leaving the preformed membrane partially separated from the egg. This involves another phenomenon.

It seems safe then to conclude that the rate of the processes leading to the development of the fertilization membrane in eggs of the four species is not limited by metabolic rate throughout most of the temperature range of fertilization. In the middle ranges it may be limited by metabolic rate.

The Q_{10} values do not indicate what the limiting reaction is, but rather, what type of reaction it could or could not be. As for the types of non-metabolic factors which limit the rate at low temperatures where the coefficients are high, e.g., $Q_{10} = 6.0$ to 12.0 and at temperatures where the coefficients are low, e.g., $Q_{10} = 1.3$ to 1.5, reasonable probabilities are viscosity in the former case and diffusion, imbibition, or some other physical factor in the latter. Viscosity, or the beginning of coagulation, may also be the limiting factor where the coefficients are negative at higher temperatures. The coefficients and their trends suggest that the underlying reactions are of the same general sort as those which limit the rate of cell division [see particularly Loeb and Wasteneys (1911), Fauré-Fremiet (1913), and in corresponding temperature ranges, certain stages in mitosis, Ephrussi (1927)].

In *Dendraster* and *Strongylocentrotus*, in contrast to *Patiria* and *Urechis*, the Q_{10} values become negative at higher temperatures. This is merely another way of describing what may be seen more directly by comparing the time-temperature curves (Fig. 1), namely, that the reaction slows up as the temperature is increased above 22° C. in the one case, while proceeding with continually increasing rate to the maximum fertilization temperature in the other. This sharp functional difference confirms the evidences already given (Chase, 1935) that there are differences in parts at least of the reactions which lead to the development of the fertilization membrane in these different kinds of eggs. It seems reasonable, and is consistent with all of the results obtained, to conclude that in *Dendraster* and *Strongylocentrotus*, in contrast to *Patiria* and *Urechis*, a relatively large measure of active transformation of a precursor substance is included in the processes which precede the development of the fertilization membrane.[3] These steps are omitted in the case of the fertilization membrane in eggs of *Patiria* and *Urechis* because there is already a well-formed membrane on the unfertilized egg which is separated after the fertilization of the egg. It may be that rate retardation above

[3] This may involve precipitation, as suggested by Harvey (1914).

22° C. is absent in *Patiria* and *Urechis* because the affected egg activities are missing. Except for the reversal in the effect of temperature on the rate at 22° C. in two of the species, the temperature coefficients suggest comparable underlying processes in the four species.

Summary

1. The activity of the spermatozoön is affected by extreme temperatures. At 2° C. the sperm of *Dendraster* and *Patiria* are feebly active while the sperm of *Strongylocentrotus* and *Urechis* are very active. The activity of the sperm increases with rise in temperature and is retarded at high temperatures. At 25° C. sperm of all species were very active, but at 28.5° C.–30° C. *Dendraster* and *Patiria* sperm became inactive. *Strongylocentrotus* and *Urechis* sperm became inactive at 35° C.–40° C.

2. At 2° C. unfertilized eggs of *Dendraster*, *Strongylocentrotus*, and *Urechis* were apparently unaffected while eggs of *Patiria* were activated by cold at temperatures of 8° C. and below. High temperatures caused partial activation of the eggs, usually followed by cytolysis. These effects occurred at and above the following temperatures: *Dendraster*, 29° C.; *Patiria*, 30° C.; *Strongylocentrotus*, 32° C.; and *Urechis*, 35° C.

3. The rate of the cortical reactions leading to the development of the fertilization membrane in eggs of the four species was retarded at low temperatures and, in the case of eggs of *Dendraster* and *Strongylocentrotus*, increased with rise in temperature up to 22° C. but decreased above 22° C. up to the maximum fertilization temperatures. In eggs of *Patiria* and *Urechis* the rate of the reaction increased steadily up to the maximum fertilization temperatures.

4. The effect of temperature on the rate of the cortical reactions in the different kinds of eggs affords a functional basis for grouping the eggs in two general categories with respect to the underlying reactions, which agrees entirely with other types of evidence in regard to the mode of the development of the fertilization membranes.

5. The temperature coefficients (Q_{10}) of the rates of the cortical reactions leading to the development of the fertilization membrane in eggs of *Dendraster*, *Strongylocentrotus*, *Urechis*, and to a lesser extent of *Patiria*, are not constant over the range of fertilization, but shift progressively over a wide range. At colder temperatures the coefficients attain values of 6.0 to 12.0 except in *Patiria*, whose eggs were not fertilized at low temperatures. At mid-temperature ranges the coefficients are 1.3 to 1.5 in the four types of eggs.

6. The coefficients indicate (see Table II) that the rate of the

cortical reaction is not limited by metabolic rate over most of the viable temperature range since over most of the range Q_{10} is not of the order of magnitude of 2.

7. It is suggested that in the cortical reactions, as in such developmental phenomena as the division of egg cells, in addition to metabolic factors, other factors such as viscosity, diffusion, imbibition, etc., are involved; and that over most of the viable temperature range, it is such factors which limit the rate of reaction.

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SEASONAL MIGRATIONS OF THE WOOD-BORER LIMNORIA LIGNORUM (RATHKE) AT FRIDAY HARBOR, WASHINGTON

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INTRODUCTION

The present report follows a year of observations instituted to verify the apparent migrations of *Limnoria lignorum* during an investigation on seasonal settlement of sessile organisms at Friday Harbor, Washington in 1928–1929 (Johnson and Miller, 1935). Since regular seasonal migrations of *Limnoria* have not before been reported, it seemed advisable to carry out at least an additional year's work as a check. Consequently, test blocks were again put in place in 1930. Some of these were lost and the work was temporarily abandoned, and further delay followed my absence from the West Coast. The ensuing lapse of time may, however, prove of value in giving data on isolated years and thus introducing more extreme conditions, at least with respect to winter temperatures.

Limnoria lignorum is a widely distributed marine wood-boring crustacean which is frequently found in vast numbers in infected areas where environmental conditions, especially salinity, are favorable to its requirements. The animals attain a maximum length of only about 5 mm., and nearly 400 individuals (juveniles predominating) may be present in one cubic inch of heavily infected wood. The deterioration of wooden marine structures due to the ravages of this gribble is an item of considerable economic importance along the Pacific and Atlantic coasts of America and in many other parts of the world where marine structures are maintained. Its attack occurs on the surface of the wood where small burrows are made, forming channels following mainly the softer, less lignified portions of the grain. Thus, as the wood becomes highly porous and friable, it is easily eroded away and deeper wood is freshly attacked producing a characteristic wasting of the timber which is most pronounced near the low tide level.

It is generally believed that the dispersal of *Limnoria* is brought about mainly by incidental transportation on infected driftwood etc., and by occasional wanderings of free-swimming individuals. The

data here given lead me to believe that the principal means of dispersal within an infected area is by a regular seasonal migration which is perhaps associated with the main breeding season. These migrations must not be understood as occurring over any considerable distances but rather as a matter of only a few meters or less. Mechanical transportation by drift of infected wood etc. still remains the only explanation for the spread of infection to more remote areas, for *Limnoria* is not a strong swimmer and does not possess the pelagic egg or larval stage which plays such an important rôle in the wide and rapid dispersal of many marine invertebrates. The eggs and young are few in numbers, about 10 to 23, and are retained in a ventral brood pouch of the female. When about 1.0 to 1.2 mm. long the young escape and immediately begin burrowing into the wood where they were born. Thus, though the reproductive power is relatively small, the hazards of a helpless pelagic existence are eliminated and the total population increases at a rapid rate. Destruction by natural enemies is apparently negligible, particularly while the animals remain in the burrows. The migrations referred to above, do, however, introduce a period of special hazard, but the short distances involved in swimming freely in the water must reduce this peril materially.

The author wishes to thank Dr. T. G. Thompson, Director of the University of Washington Oceanographic Laboratories, for laboratory facilities and equipment at Friday Harbor; and Dr. Lyman D. Phifer, Assistant Director, for 1934 temperature data.

Methods

Seasoned Douglas fir boards, 9 cm. × 2 cm. × 35 cm., served as test blocks. Prior to being used, these were soaked for two weeks in sea water which had been heated to kill all larvæ. A set of two blocks constituted a series. The two blocks were attached one meter apart to a long wire which was suspended vertically from a beam under the Friday Harbor wharf where there was known to be a heavy infection of *Limnoria*. A weight attached to the wire below the lower block held it in position so that the upper block was a little above zero tide level and the lower block somewhat below this level (In the 1928–29 investigation it was found that nearly 90 per cent of the *Limnoria* settle at these levels). Horizontally the blocks were about a meter from the nearest piles.

The number of animals occurring on an infected block was determined by counting the burrows, and when infection was light all the burrows were counted, but during heavy attack only a limited portion was counted and the numbers computed from the size of the areas involved.

In order to obtain a census of size distribution which would be typical of the whole population, and not a selected one as appears to exist on newly populated test blocks, each month a small sample of wood was taken from an old infected wharf pile. These samples included a clean cut from the surface to unattacked wood. The wood thus removed was picked apart and all sizes of *Limnoria* recorded from the sample selected. In this operation it was necessary to use a dissecting microscope, for the young stages are quite colorless and can be seen against the wood only with difficulty.

The sexes are separable only on minute differences, hence in the examination for gravid females as an index to reproductive activity, no separation of sexes was made and all specimens of adult size (3.4 mm. and over) were considered.

It should be noted that the test pieces were always in a shaded position and, aside from a few filamentous diatoms, no vegetation which would interfere with settlement occurred on the wood. A number of sessile animals, mainly hydroids, bryozoa, barnacles, and tube worms were found particularly in the spring and autumn, but not in numbers sufficient to interfere with the activities of *Limnoria*.

TABLE I

Seasonal settlement of Limnoria lignorum in two-month periods from September 5, 1933 to September 8, 1934

Period of exposure	Sept. 5 to Nov. 7	Nov. 7 to Jan. 5	Jan. 5 to Mar. 7	Mar. 7 to May 9	May 9 to July 7	July 7 to Sept. 8
Upper block	8	32	348	605	146	16
Lower block	16	41	314	437	149	26
Totals	24	73	662	1,042	295	42
Percentage of yearly settlement	1.12	3.42	31.00	48.80	13.80	1.97

RESULTS

Examination of the test blocks which were exposed to settlement and attack over periods of two and three months showed a marked increase in the number of *Limnoria* settling on the blocks during late winter and spring months. The remaining portion of the year was relatively free from settlement but there was no period of complete immunity.

Two-month Series.—The seasonal distribution as indicated by the number of animals occurring on the test blocks at the end of each two months of exposure is given in Table I. A study of these data shows

that the first significant increase in settlement occurred sometime between January 5 and March 7, when the blocks for this period were removed. The next two months, March 7 to May 9, witnessed a yet greater settlement, as is evidenced by examination of the blocks removed May 9 when the greatest number of individuals was counted. During the next period, ending July 7, there was a definite decrease in numbers. The decline continued to the end of the investigation on September 8. The period of minimum migration covers September and October, when only 1.12 per cent of the total yearly settlement took place, but July, August, November and December also witness reduced migratory activity. The maximum migration may be assumed to have occurred between the dates having the highest counts, that is, some time in March or April, the two-month period having

TABLE II

Seasonal settlement of Limnoria lignorum in three-month periods from September 5, 1933 to September 8, 1934

Period of exposure	Sept. 5 to Dec. 6	Dec. 6 to Mar. 7	Mar. 7 to June 9	June 9 to Sept. 8
Upper block	18	572	1,316	118
Lower block	25	335	1,400	207
Totals	43	907	2,716	325
Percentage of yearly settlement	1.08	22.65	68.00	8.15

48.80 per cent of the yearly settlement. It is instructive to note that 93.60 per cent of the total settlement took place over the six-month period, January 5 to July 7, and that 79.80 per cent was for the four months from January 5 to May 9. The numbers recorded when the blocks were removed and examined represent a two-month accumulation. Hence, when these blocks cover a period at the beginning of increasing migration, it is reasonable to believe that the greatest number of *Limnoria* settled on the blocks toward the end of that period. Conversely, on a waning migration, after the maximum is just past, the first portion of the period following would receive the greater number, provided decline be uniform. Thus the total period over which migration was most pronounced is somewhat narrowed, and January and also June become much less important than February and May, the two months which were included with them respectively in the periods of exposure to settlement immediately before and after the crest of the migration.

Three-month Series (Table II, Fig. 1).—These show essentially the

same as the two-month series. The longer period of exposure makes an interpretation of the data more difficult and uncertain, but they indicate a continuance of rather active migration through May. By reference to the two-month series, it will be noted, however, that the month of May did not experience a very heavy settlement and it is probable that that month contributed only moderately to the maxi-

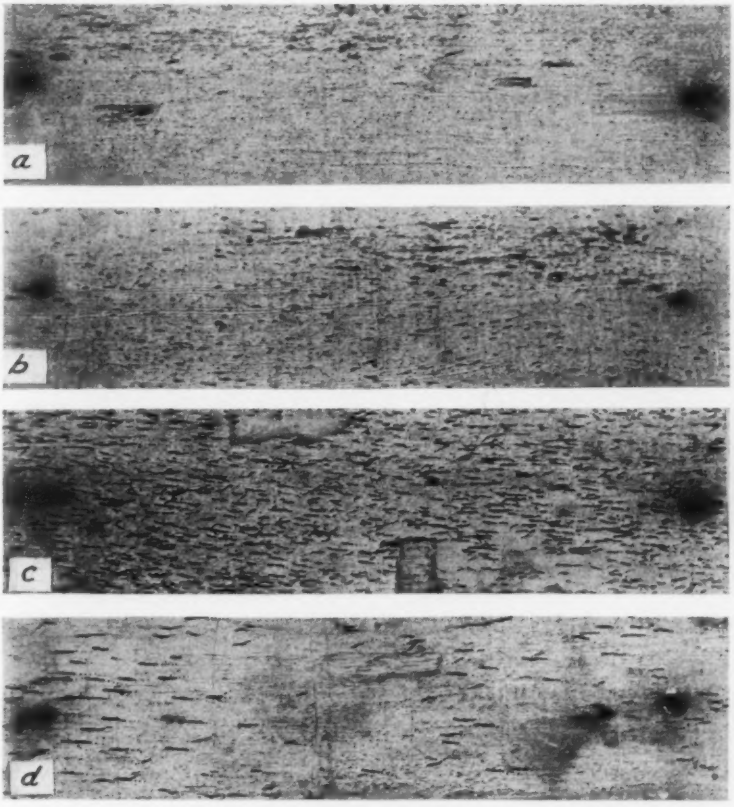

FIG. 1. The lower blocks of the four series which were exposed to settlement of *Limnoria lignorum* over the three-month periods indicated:

a. September 5, 1933, to December 6, 1933;
b. December 6, 1933, to March 7, 1934;
c. March 7, 1934, to June 9, 1934;
d. June 9, 1934, to September 8, 1934.

mum which occurred from March 7 to June 9. December is similarly but more completely eliminated as an important month in the period December 6 to March 7, with the result that most of the 90.65 per cent of settlement which occurred on the exposures covering the six months from December 6 to June 9 must have taken place during January, February, March, April, and May, with the importance of January doubtful, and May more or less restricted.

A comparison with the three-month series of 1928–29 (Johnson and Miller, 1935) shows in general a very close agreement. During that year, however, pronounced migration appears to have begun a little later in the winter and continued later in the spring, making May also an important migratory month and to a much lesser degree also June. In selecting (from the over-lapping series of 1928–29) a succession of series covering three-month periods from October 1, 1928 to September 26, 1929, we find the following percentage of total yearly settlement for the periods indicated: October 1 to January 1, 1.98 per cent; January 1 to March 28, 27.93 per cent; April 1 to July 2, 63.92 per cent; July 2 to September 26, 6.17 per cent. From this it is seen that 91.85 per cent of the total yearly settlement occurred over the six-month period from January 1 to July 2. It is obvious, however, that January was not an important migratory month that year and contributed but little to the total settlement of the six-month period.

In 1928–29 active migration occurred on a rising temperature which ranged from about 6.55° C. to 9.99° C. In 1933–34 the average monthly temperature range over the most active period was from 7.71° C. to 9.42° C. The minimum temperature recorded for 1929 was on February 1, at which time it was 6.4° C., whereas in 1934 a minimum of only 7.52° C. was recorded on February 22. The lower temperatures of 1928–29 may be responsible for the tendency to later migration during that year (See Table V). Salinities at Friday Harbor are always within the limits required by *Limnoria* (Kofoid and Miller et al., p. 327, 1927).

Size Groups.—Measurements of specimens occurring on the test blocks revealed only mature or submature animals except in a few instances where also very young specimens were recorded from the three-month blocks removed June 9 and September 8. These young were, however, few in number and doubtless were from broods recently released.

The ratio of young to the intermediate and adult groups gave a very different picture in the samples which were collected from the population of old infected wharf-piles. Three size groups were considered, namely, 2.0 mm. or less; 2.1 mm. to 3.4 mm.; 3.5 mm. or over.

The first group comprises recently or newly hatched young, the second group, older juveniles and possibly some small mature males, and the third group, only adults. From Table III it will be seen that the youngest group was the dominant one throughout the year (no sample available for August). During late summer and early winter there is a gradual relative decrease as the specimens of this group become older and enter into the group of intermediate size. During March, (April?) and May, however, the percentage of very young is noticeably increased. The adult group remains relatively constant, apparently as

TABLE III

Limnoria lignorum.—Size groups from old wharf-pile population

Date	Total no. examined	2 mm. or less	Percentage of total	2.1 mm. to 3.4 mm.	Percentage of total	3.5 mm. or over	Percentage of total
1933							
Oct. 5	103	65	63.11	20	19.41	18	17.48
Nov. 7	127	62	48.88	32	25.20	33	25.94
Dec. 6	206	104	50.48	64	31.07	38	18.44
1934							
Jan. 5	101	50	49.50	35	34.65	16	15.84
Feb. 8	251	114	45.42	97	38.64	40	15.93
Mar. 7	291	172	59.10	86	29.55	33	11.34
Apr. 7	441	187	42.40	126	28.60	128	29.00
May 9	348	251	72.12	37	10.63	60	17.24
June 9	125	72	57.60	36	28.80	17	13.60
July 7	97	56	57.73	14	14.43	27	27.83
Aug.	—	—	—	—	—	—	—
Sept. 8	229	123	53.71	66	28.82	40	17.46

a result of animals passing over from the intermediate group at all seasons at a rate which masks any decline which might otherwise be apparent due to death rate. Since the trend of the percentages of the youngest group is a function of the other two groups, it is influenced by such changes as death rate of the adults, or migration of the older members of the intermediate group, but the relationship which it bears to the occurrence of gravid females indicates that it must also represent actual population changes in the group.

Gravid Females.—A record of the seasonal distribution of specimens carrying eggs or young in the brood pouch is given in Table IV. Gravid specimens were found only in the group measuring over 3.4 mm. long. It is evident that gravid females do occur throughout the year, that few are found in January and February, and that especially large numbers are gravid in early April and May, and the data suggests

also a small increase in November and December. In 1928–29 it was also found that gravid specimens were present in each month of the year, with a minimum in February and early March and with significant numbers through spring, summer, and autumn. In that year there was indicated a rise following the main migratory season. In 1933–34 the reproductive activity appears to be more closely correlated with the maximum migratory activity but still somewhat lagging. The low temperatures of February and March of 1929 may account for the scarcity of gravid females in February and early March of that year,

TABLE IV

Limnoria lignorum—Gravid females

Date	Population from old wharf-piles		Population from two-month test blocks		Population from three-month test blocks		Total examined all populations	Percentage of gravid females in adult group
	No. adults examined	No. gravid females	No. adults examined	No. gravid females	No. adults examined	No. gravid females		
1933								
Oct. 5	55	6	—	—	—	—	55	10.91
Nov. 7	33	11	16	3	—	—	49	28.57
Dec. 6	38	11	—	—	16	4	54	27.78
1934								
Jan. 5	16	1	26	0	—	—	42	2.39
Feb. 8	40	3	—	—	—	—	40	7.50
Mar. 7	33	4	10	0	10	3	53	13.20
Apr. 7	101	49	—	—	—	—	101	48.50
May 9	60	18	45	5	—	—	105	21.90
June 9	17	3	—	—	65	18	82	25.60
July 7	27	4	38	9	—	—	65	20.00
Aug.	—	—	—	—	—	—	—	—
Sept. 8	40	13	23	5	60	11	123	23.58

whereas in 1934 the fewest gravid specimens were found in January and February, the two months of lowest average temperature during that year.

Kofoid (1921, p. 52) found eggs and young throughout the year in San Francisco Bay, but no definite breeding season was established. It was suggested later (Kofoid and Miller et al., 1927, p. 329) that this lack of a definite reproductive season may be due to the small seasonal variation in temperature, i.e., approximately 8° C. Coker (1923), working at Beaufort, North Carolina, reports a definite breeding season which is correlated with a temperature of 14° C. At Beaufort the temperature range is given as about 20° C. At Friday Harbor the temperature range is only about 7° C. Henderson (1924, p. 321)

observed gravid females at St. Andrews, New Brunswick, during July, August, September, October, and to a much lesser extent also in November and December. The other months were not investigated.

Discussion

From the above data it is quite evident that we are dealing with a period of active migration of *Limnoria*. The test blocks could be reached only by swimming animals or by chance transportation on driftwood or tidal currents. The orderly distribution through the seasons on several isolated blocks, and the uniformity of age in the population excludes the possibility of chance.

This migration appears to be a preliminary or complementary activity associated with the main reproductive season. It is reasonable to believe that, unless the death rate nearly equals that of reproduction,

Table V

Temperature ranges at Friday Harbor during most active migratory period of Limnoria lignorum in 1929 and 1934

1929	Maximum °C.	Minimum °C.	Monthly average	1934	Maximum °C.	Minimum °C.	Monthly average
Jan.	7.8	7.1	7.53	Jan.	7.76	7.61	7.70
Feb.	6.7	6.4*	6.55	Feb.	7.82	7.52†	7.71
March	7.4	6.8	7.12	March	8.50	7.70	8.02
April	8.0	7.5	7.80	April	9.22	8.20	8.71
May	9.7	8.5	8.97	May	10.28	8.82	9.42
June	10.6	9.4	9.99	June	11.78	9.98	10.69
July	11.5	10.2	10.75	July	11.42	10.10	10.66

* February 1. † February 22.
1929 data from Thompson and Johnson 1930.

some movement of population must take place when the numbers become intolerable for an area which is constantly being diminished by the ravages of the population. During this migration many individuals no doubt move to already infected wood. Nevertheless there would be a general thinning out in the densely populated communities, and a large number of the migrants and their progeny would become established in more favorable habitats on sparsely populated or new timbers. The migrants represent that element of the population which is approaching or has just reached maturity, and but few, if any, of these appear to be gravid at the time of settling on the new habitat. This is brought out by the relatively small number of gravid specimens found on the two-month test blocks and also by the relative scarcity of very young

animals even on the three-month blocks. Temperatures at Friday Harbor during the most active migratory period (See Table V) were perhaps sufficiently low to induce a slow incubation, but two months, or at any rate three months would doubtless suffice for incubation, and one would expect to find large numbers of young on the blocks had the females been gravid at the time of settling.

Kofoid and Miller et al. (1927, p. 320) found a very regular and uniform infection of untreated test timbers exposed to *Limnoria* settlement in San Francisco Bay. This was true during every month of the year, and long exposures showed regular increasing severity of attack. They call attention to the problematic nature of the methods of dispersal but do not consider free-swimming a factor of much importance. In extensive tests of the reactions of *Limnoria* to wood and wood extractives, they found no evidence of a chemotropism which would aid the swimming animals in locating and recognizing submerged wood. Perhaps such a chemotropic mechanism is actively functional only at the age when active migration occurs.

Investigations in other areas also have not given evidence that local dispersal of *Limnoria* is due mainly to seasonal migrations. This may indicate an absence of pronounced seasonal variation in migratory activity in the localities investigated. There is, however, also some likelihood of the activity being overlooked, for studies pertaining to *Limnoria* have often been incidental to general wood-boring investigations where methods were designed to give information mainly on the molluscan borers or simply to determine the durability of different woods or preservatives. The effect of seasonal migrations may go unnoticed as a result of allowing test boards to remain in the water so long that population increases have occurred due to reproduction by the animals established on the boards. Thus the numbers of those actually settling on the boards as migrants would become masked by the large numbers resulting from reproduction, and also migrants may become discouraged from settling on already heavily populated wood. It is conceivable that the effect of swimming migration may also be largely obliterated by animals crawling from infected wood to conveniently placed test blocks. Commonly, test pieces are placed directly in contact with already infected wood, which may be a wharf-pile or a frame of wood specially constructed to support successive series of test boards.

Much effort has been directed towards devising means of protecting wooden piles and other marine structures against the devastating attacks of this and other marine borers. Creosote and various paints or surface applications offer a greater or lesser degree of protection, but at best this is not absolute or permanent, and because of the expense in-

volved in utilizing creosoted timbers, many marine structures are built of untreated materials, and may receive little or no subsequent protective treatment. Such structures are necessarily short-lived in areas where borers are abundant.

From the foregoing data on the migratory habits of *Limnoria*, one is led to believe that when the nature of wooden marine structures does not preclude treatment, the most efficient time to make a protective application would be at the beginning of the main migratory season. Thus the minimum amount of leaching of the protective coating would have occurred before the period when it is most needed to repell the invasion of the animals. Other things being equal, unprotected timbers submerged in June or July should show much less deterioration at the end of a year than similar timbers submerged in February or March. The latter would suffer not only an early intensive attack of the migrants, but later also that of their young. The former, on the other hand, would have a six-month period of lighter early attack and subsequently fewer young produced to consume the wood over the last half of the year.

Summary

1. Wooden test pieces were exposed to settlement of *Limnoria lignorum* in successive two and three-month periods from September 5, 1933 to September 8, 1934.

2. Monthly collections of wharf-pile populations were examined to determine the seasonal fluctuations in age groups, and in the occurrence of gravid females.

3. A marked seasonal variation in the numbers of *Limnoria* settling on the test blocks was found, and since the blocks could be reached only by swimming animals, it is believed to represent a definite seasonal migration which is associated with the main breeding season.

4. On the two-month test pieces, 93.60 per cent of the total yearly settlement of *Limnoria* occurred over a six-month period from January 5 to July 7; and 48.80 per cent settled from March 7 to May. 9. The three-month pieces showed essentially the same distribution.

5. The most active migration took place during a temperature range of about 7.71° C. to 9.42° C.

6. There was a predominance of young *Limnoria* throughout the year, but the greatest percentage of these was evident during the spring months.

7. Gravid females doubtless occurred throughout the year (no data for August), with a maximum in April and May and a minimum in January and February, the two months with the lowest temperatures.

8. As an aid to better protection against *Limnoria* attack on submerged timbers, it is suggested that the most effective time to apply protective paints etc. is at the beginning of the main migratory season.

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FEEDING HABITS AND PHARYNGEAL STRUCTURE IN STENOSTOMUM

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Introduction, Materials and Methods

A comparative study of pharyngeal structure was suggested by the diverse feeding habits exhibited by certain species of *Stenostomum*. The species selected for study were *S. grande* Child (1902), *S. virginianum* Nuttycombe (1931) and *S. predatorium* Kepner and Carter (1931).

S. grande typically feeds upon relatively large masses of debris which may contain bacterial glea, protozoa, rotifers, unicellular plants, and the like. It will also occasionally ingest whole, smaller specimens of its own or other species of *Stenostomum*. The mouth is capable of enormous distension. The food taken into the pharynx is retained momentarily and then passed through the pharyngo-enteric sphincter into the enteron.

The food of *S. virginianum* consists almost entirely of small, motile animals such as protozoa, rotifers, and whole, smaller members of its own or other species of *Stenostomum*. In the capture of its prey the mouth is quickly opened and the pharynx is dilated almost simultaneously. The suction created by the dilation draws the prey into the pharynx where it is imprisoned by the contraction of the oral sphincter. Usually the captured organism is retained in the pharynx for several seconds before it is forced back into the enteron by the simultaneous opening of the pharyngo-enteric sphincter and contraction of the intrinsic, circular musculature of the pharyngeal wall.

S. predatorium appears to exist entirely upon the tissues of other species of *Stenostomum*. We have for several years maintained cultures of this species by feeding it upon *S. tenuicauda*. *S. predatorium* feeds in a peculiar manner. It applies its "lips" to the surface of *S. tenuicauda* and, through the combined "sucking" action of its pharynx and constriction of its oral sphincter, is capable of drawing in and pinching off pieces of its prey which may then be passed into the enteron. It seldom, if ever, ingests a whole animal as do both *S. grande* and *S. virginianum*. Instead it takes a small piece of its prey whenever it comes in contact with it.

Thus we have here three species which differ sharply in feeding habits and which we have found to exhibit a high degree of morphological, pharyngeal adaptation.

Representatives of the three species were studied in the living condition and in fixed preparations. For fixation Gilson's and modified (double quantity of osmic acid with addition of 10 per cent urea by weight) Meeve's fluids were used. Fixed material was embedded in rubber-paraffin and sectioned at 5 micra. Heidenhain's haematoxylin was used as a stain.

General Morphology of the Pharynx

The pharynx in the genus *Stenostomum* is essentially a simple, tubular structure equipped at both oral and enteric ends with sphincter muscle fibers. The intrinsic musculature of the pharyngeal wall is composed of circular and longitudinal layers. Extrinsic, radial muscle fibers anchor the pharynx to the body wall. In most species unicellular glands are associated with the pharyngeal region.

Comparative Morphology

Comparison of Living Specimens.—Studies of the pharynx in living specimens of these three species of *Stenostomum* show sharp differences in gross morphology.

In *S. grande* (Plate I, Fig. 1) the pharynx is marked off rather definitely by a transverse constriction into an anterior region, which is covered externally by unicellular glands, and a posterior (œsophageal) region lacking glands. The radial musculature in this species is relatively undeveloped and consists of scattered fibers running from the pharyngeal wall to the body wall.

Explanation of Plate I.

Fig. 1. Dorsal aspect of pharyngeal region of living *S. grande.*
Fig. 2. Dorsal aspect of pharyngeal region of living *S. virginianum.*
Fig. 3. Dorsal aspect of pharyngeal region of living *S. predatorium.*
Fig. 4. Transverse section through the anterior pharyngeal region of *S. grande.*
Fig. 5. Transverse section through the mid-region of the pharynx of *S. virginianum.*
Fig. 6. Transverse section through the posterior pharyngeal region of *S. predatorium.*

List of abbreviations used in Plates I and II: *a*, special oral sphincter; *b*, cerebral commissure; *c*, circular muscle layer of the pharynx; *d*, concentrated mass of radial fibers; *e*, epidermis; *en*, enteron; *ep*, œsophageal portion of the pharynx; *f*, free-cells of the pseudocœl; *g*, pharyngeal gland cells; *h*, intrinsic musculature (longitudinal and circular); *l*, longitudinal muscle layer of the pharynx; *m*, mouth; *n*, protonephridial tube; *o*, oral sphincter; *oe*, oral epithelium; *p*, pharynx; *pe*, pharyngeal epithelium; *ps*, pharyngo-enteric sphincter; *r*, radial muscle fibers; *s*, special, heavy, radial fibers; *t*, testis. Epidermal cilia and rhabdites omitted in all figures.

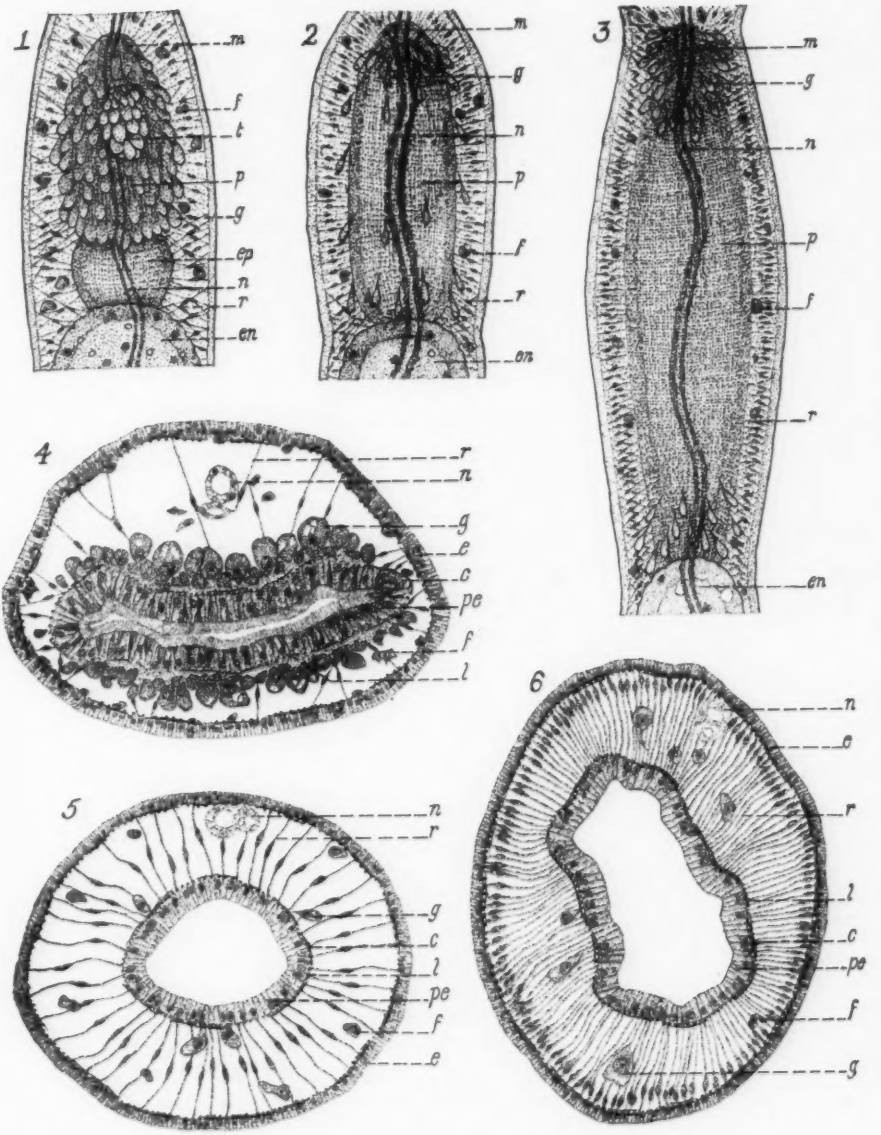
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PLATE I

In *S. virginianum* (Plate I, Fig. 2) the pharynx shows no regional differentiation of its wall and is of rather uniform diameter throughout its length. The unicellular glands are smaller and are distributed sparsely over the entire surface of the pharyngeal wall. The radial musculature is much more elaborate than that of *S. grande*.

S. predatorium (Plate I, Fig. 3) has a relatively much longer pharynx than either of the preceding species. Its wall shows no regional differentiation. Large unicellular glands are in this species confined to the oral and enteric ends of the pharynx. The radial musculature is the most elaborate found in the American species of the genus—the fibers are so heavy and so closely packed as to almost obscure the pharynx which they support. In the figure only the lateral, radial fibers are shown.

Comparative Histological Structure.—Transverse and sagittal sections of the pharyngeal region of the three species clearly illustrate the main histological differences.

Transverse and sagittal sections of this region in *S. grande* are shown respectively in Plate I, Fig. 4, and Plate II, Fig. 1. The transverse section was taken through the anterior pharyngeal region. The ciliary coat of the pharyngeal epithelium (*pe*) is extremely dense in this region and the epithelium itself shows groups of chromatic lines. Just outside the basement membrane of the epithelium may be seen the cut ends of the fibers (*l*) composing the longitudinal layer of the intrinsic, pharyngeal musculature and just external to this layer lies the circular layer (*c*) of the intrinsic pharyngeal musculature. The bundles of the longitudinal layer are considerably heavier than those of the circular layer. Lying in the pseudocœl may be seen the numerous, large, unicellular, pharyngeal glands (*g*) characteristic of this region. The radial, pharyngeal musculature is composed of a few relatively weak fibers (*r*) passing from the wall of the pharynx to the body wall. These fibers frequently show branching. The sagittal section of *S. grande* brings out some additional features. The epithelium of the posterior (œsophageal) pharyngeal region (*ep*) is thinner and its ciliary coat much less dense. There is not any great concentration of circular muscle fibers in either the oral or the pharyngo-enteric sphincter regions. The points of attachment of the radial muscle fibers are more numerous just back of the constriction representing the line of demarcation between the anterior and œsophageal, pharyngeal regions. Associated with the dorsal lip may be seen the bases of a concentrated mass (*d*) of radial fibers. The distal ends of some of these fibers are attached dorsally and laterally to the body wall at the level of the mouth and still others are attached to the body

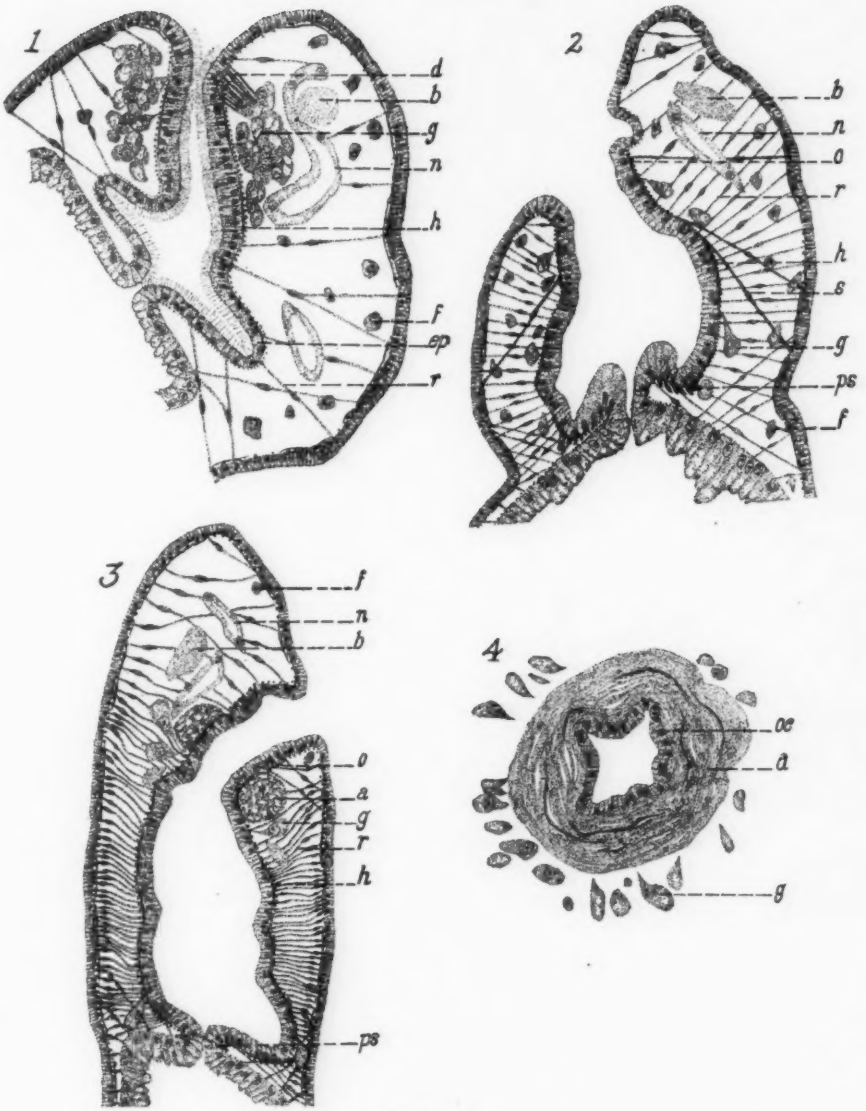

PLATE II

FIG. 1. Sagittal section through the pharynx of *S. grande*. Testis not shown.
FIG. 2. Sagittal section through the pharynx of *S. virginianum*.
FIG. 3. Sagittal section through the pharynx of *S. predatorium*.
FIG. 4. Slanting, frontal section through the special, oral sphincter of *S. predatorium*.

wall at more posterior levels. The distal attachments do not show in the section illustrated. These fibers are responsible for the extreme extensibility of the oral opening.

The transverse section (Plate I, Fig. 5) through the middle of the pharynx of *S. virginianum* gives us a very different picture. The ciliary coat of the pharyngeal epithelium (*pe*) is so fine as to appear lacking in many sections. The most significant differences, however, are seen in the radial musculature and pharyngeal glands. The radial muscle fibers (*r*) in *S. virginianum* are numerous and well-developed. The pharyngeal glands (*g*) are few and small. The sagittal section (Plate II, Fig. 2) of this species shows to even better advantage the distinctive features of its pharyngeal structure. The pharyngeal epithelium is of uniform thickness throughout the length of the pharynx. The intrinsic, longitudinal musculature shows about the same degree of development as in *S. grande* but the intrinsic, circular layer shows unusually large bundles in both the oral (*o*) and pharyngo-enteric sphincter (*ps*) zones. The radial muscle fibers (*r*) are numerous and powerful. In addition to the regular radial fibers there are several unusually heavy bundles (*s*) so placed as to be capable of making possible a greater extension of the oral opening. The small pharyngeal glands (*g*) are not localized in the anterior half of the pharynx as are the corresponding but larger glands in *S. grande*.

The transverse section (Plate I, Fig. 6) near the posterior end of the pharynx of *S. predatorium* shows an epithelium (*pe*) which is apparently unciliated. The intrinsic, pharyngeal musculature at this level is developed to approximately the same extent as is the corresponding musculature in *S. grande* and *S. virginianum*. The radial musculature, however, is enormously more highly developed than that of either of the other species. The nuclei of the radial fibers (*r*) are not located indiscriminately but are placed near the point at which the fiber is inserted on the body wall. The large, unicellular, pharyngeal glands (*g*) are prominent features at this level of the pharynx. The sagittal section (Plate II, Fig. 3) of *S. predatorium* shows a uniform, pharyngeal epithelium. The intrinsic, pharyngeal musculature shows some strengthening in its circular bundles at both the oral (*o*) and pharyngo-enteric sphincter (*ps*) regions. In addition to the strengthened bundles of the regular circular layer at the oral level there is an extremely powerful, special, oral sphincter (*a*). This ring of muscle surrounds the anterior opening into the pharynx and can be seen in sagittal section as a mass above and below the oral end of the pharynx. A slanting, frontal section of this sphincter is shown in Plate II, Fig. 4. This special sphincter is the most powerful mus-

cular unit occurring in any of the American species of the genus. The sagittal section also shows the extremely powerful, radial musculature (*r*). The large, unicellular glands (g) are confined to the anterior and posterior pharyngeal regions.

Summary

The variations in feeding habits exhibited by different species of the genus *Stenostomum* are largely dependent upon adaptive modification of pharyngeal structure.

The pharynx of *S. grande* is well adapted to the ingestion of large masses of relatively inert food material. The heavy band of radial muscles attached at the lip region is so arranged that it is capable of causing the enormous distension of the oro-pharyngeal region thus making possible the ingestion of very large masses. The heavy ciliary coat of the anterior, pharyngeal region is adapted to aid in the backward propulsion of such large masses into the thin-walled, distensible, œsophageal region of the pharynx from whence the mass may be forced through the pharyngo-enteric sphincter by the activity of the intrinsic, pharyngeal musculature.

In *S. virginianum*, the food of which is largely made up of small, highly motile prey, we find well-developed oral and pharyngo-enteric sphincters which allow the imprisonment of the captured organisms in the pharynx. There are several large bundles of radial fibers so disposed as to make possible a quick distension of the lips, and the sudden contraction of the powerful, radial, pharyngeal musculature enables this species to generate sufficient suction to draw its prey into the pharynx.

The pharynx of *S. predatorium* shows two prime structural adaptations. The radial musculature is strong enough to produce sufficient suction to draw into the mouth the tissues of another species of *Stenostomum* and the special oral sphincter by its contraction is capable of pinching off from the body of the prey the mass of tissue drawn into the pharynx.

In general it may be said for all species that the extrinsic, radial musculature primarily determines the method by which the food is drawn into the pharynx and the general intrinsic musculature of the pharyngeal wall is responsible for the transmission of the food to the enteron.

We have been unable to assign any differential, functional capacity to the pharyngeal glands in spite of the fact that sizes and distribution in the three species are sharply different.

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MITOSIS IN AMŒBA DUBIA[1]

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Since the first accurate description of the two common, large, free-living amœbæ, *Amœba dubia* and *Amœba proteus*, by Schaeffer (1916) very little has appeared in scientific literature concerning the process of mitosis in either of these species. The only reasonably complete account is that of Chalkley and Daniel (1933) in which the nuclear phases of division in *Amœba proteus* are described. Awerinzew's paper (1904) is much too brief and indefinite to give an adequate picture of the process and it is certainly not established that his work was done on *Amœba dubia*. The figures of Carter (1912) are unlike any phase seen by us in this work. Furthermore, certain phenomena observed by the writers have not yet been described in any account previously published concerning division in free-living amœbæ.

The senior author has been interested for a considerable time in specific differences between *Amœba proteus* and *Amœba dubia*. Differences in their structure and physiology have already been noted (Dawson and Belkin, 1929). As the nucleus of *Amœba dubia* is morphologically very different from that of *Amœba proteus*, it was thought that a study of mitosis in the former would reveal still further specific differences.

Material and Methods

The material used was a strain of *Amœba dubia* descended from a single individual which was isolated from a culture collected in a pond in the vicinity of Woods Hole, Mass., in 1926. This strain and others of the same origin have been cultivated continuously by the senior author since that time. An account of the method of culture has been previously published (Dawson, 1928). With but slight modification this method has been used in obtaining dividing animals for this work.

[1] The authors wish to express their indebtedness to Dr. James Kendall of the College of the City of New York for his kind assistance in preparing the photomicrographs used in this paper.

It was found relatively simple to obtain cultures in which forty to fifty individuals in all phases of division could be isolated in the course of a single afternoon. The dividing amœbæ were isolated from these cultures by means of a Spencer binocular dissecting microscope using transmitted light.

The organisms to be stained were isolated by means of a capillary pipette of appropriate size and placed on a coverslip with a minimum amount of culture medium. The following fixatives were used: Schaudinn's fluid, strong both with and without acetic acid, Bouin's (the B–15 modification of Allen), strong Flemming's and Carnoy and Lebrun's fluid. Of all these fixatives the one most used was Carnoy and Lebrun's fluid. The picture was essentially the same with the various fixatives. Carnoy and Lebrun's fluid has the great advantage of making the fixed amœbæ adhere to the coverslip, thus greatly facilitating the staining and clearing process. Specimens were usually taken up through the various alcohols and staining and clearing solutions on the coverslip although a number were carried up in the solutions while floating loose in a small culture dish.

The following stains were used: Heidenhain's hematoxylin (Grübler's); gentian violet according to the technique of Johansen (1932). This method is rapid and is particularly useful for comparison of series of preparations. We also used picrocarmine, which is exceptionally good for clarity of detail since it stains chromatin well, giving a good contrast with other structures. The larger achromatic bodies stained very slightly with this stain and the chromatin was a clear red. Delafield's hematoxylin was used occasionally, and although a good general stain, showed no significant features not shown by other stains. Of all these the stain most generally used was Heidenhain's hematoxylin. Both long and short methods of staining were employed. Differentiation was done with iron alum and also in other cases with a saturated aqueous solution of picric acid following the method recommended by Dr. G. W. Kidder (1933). In general, it was found that Heidenhain's hematoxylin gave a clearer picture of mitotic phenomena than any other single stain. Although much more difficult to operate due to danger of loosening the animal, the picric acid-ammonia method of differentiating was found to be superior to the iron alum treatment.

The Feulgen method for differentiation of chromatin was used. Feulgen's procedure as given in McClung's Handbook of Microscopical Technique (p. 483) and the modification presented in Lee's Handbook (p. 306, ninth edition, 1928) were employed. In some cases Feulgen preparations were counterstained with light green. Staining for volutin granules in various stages of the vegetative cycle of *A. dubia* was done. The standard technique for volutin staining was used.

Amœbæ in vegetative phases and in division phases were imbedded in paraffin and sectioned. Sections 4 to 8 μ in thickness were cut. The method of imbedding was similar to that used previously by the senior author (Dawson, 1926). In some cases amœbæ were stained, differentiated and then removed from the coverslip and imbedded and sectioned.

Nuclear Structure of Vegetative Amœba

As described by Schaeffer (1916) the nucleus of *Amœba dubia* is "a prolate spheroid; chromatin distributed in several thousand masses of from one and one half to two microns immediately under the nuclear membrane; size of nucleus about 40 μ by 32 μ short diameter." He makes no mention of the internal structure of the nucleus in this species. In a later paper (1926) the same author classes the nuclei of *Amœba proteus* (*Chaos diffluens*) and *Amœba dubia* (*Polychaos dubia*) under the same general type and states that in this type the chromatin occurs in the living 'resting nucleus' "in a layer of small grains of uniform size at a greater or less distance from the nuclear membrane." The illustrations of this type show invariably a clear space in the central part of the nucleus. Chalkley and Daniel (1933) say that "in the nucleus of *A. proteus* the chromatin, which in the resting nucleus is distributed in granules or 'blocks' immediately beneath the nuclear membrane, leaves this position and moves toward the center of the nucleus." Doflein (1918) describes the chromatin as arranged under the nuclear membrane and also a definite endosome centrally placed in the nucleus of *Amœba proteus*. Taylor (1923) figures the nucleus of *A. proteus* with a karyosome and peripherally arranged chromatin granules under the nuclear membrane. One may conclude from these and other sources that, in the nucleus of *A. proteus*, the chromatin is arranged peripherally under the nuclear membrane and that an endosome (karyosome) may or may not be present.

The nuclear membrane of *Amœba dubia* is clearly defined and can easily be seen in both living and stained preparations. In the stained vegetative nucleus a uniform distribution of staining granules approximating the size of those described by Schaeffer occurs throughout. Our measurements of the staining granules taken from sectioned nuclei give a size range of from 0.2 to 1.7 μ in diameter. In examining a total mount of a vegetative or 'resting' amœba, a peripheral arrangement of staining granules in the nucleus seems to be indicated. This, however, is an optical illusion due to the appearance of these granules in the optical axis. By careful focussing one may determine that the granules are regularly arranged throughout the entire nucleus. That this is a fact is shown clearly by serial sections of the vegetative nucleus (Figs.

1, 21, *a–g*). In the sections the granules do not seem to be connected and the matrix in which they are imbedded is achromatic. No evidence of a fibrillar structure of the matrix was found in any of our sections. Feulgen preparations of vegetative amœbæ were largely negative. In some cases it was possible to detect a slight pinkish color in the granules, presumably chromatin, of the nucleus; this, despite the fact that in the same preparation the nuclei of ciliates gave the typical Feulgen reaction.

The Process of Fission as Seen in the Living Amœba

The various phases of fission in *Amœba dubia* (called by the author *Amœba proteus*), in respect to changes of cytoplasmic form only, have been described by Botsford (1926). There are no outstanding differences of form between Botsford's figures for *A. dubia* and Chalkley and Daniel's for *A. proteus*. We also have found that this is true. Botsford has stated that she found it impossible to observe either nucleus or contractile vacuole while the amœba was in the so-called 'division sphere.' Chalkley and Daniel, on the other hand, noted in *A. proteus* a 'hyaline area' which persisted during the greater part of the division process and only disappeared during the late anaphase. This hyaline area, according to these authors, indicated the position of the nucleus. In the division sphere of *Amœba dubia* we found, as Botsford did, that it was impossible to observe any trace of the nucleus. No hyaline area has ever been seen by us. The earliest stage in fission in *Amœba dubia* shows a general rounding of the periphery and a shortening of the pseudopodia throughout. At this stage the amœba, resting on the glass surface of the container, is slightly thicker than usual. Within a few minutes the organism becomes almost completely spherical—the so-called 'division sphere'—extremely opaque and the pseudopodia become much shorter. We have found that the division sphere persists practically unchanged for a period which varies from fifteen to twenty minutes (Plate I, Fig. 2, *a* and *b*, and Fig. 3). As the division process con-

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Explanation of Plate I

Fig. 1. *Amœba dubia* Schaeffer. Total mount. × 1350. View of vegetative form showing nuclear structure and two food vacuoles.

Fig. 2. Division spheres in *Amœba dubia* Schaeffer. *A*. Total mount showing anaphase (barrel stage). Note spherical form and relatively short regular pseudopodia. *B*. Total mount showing early telophase. Note similarity of division sphere and pseudopodia. *A* and *B*. × 450.

Fig. 3. *Amœba dubia* Schaeffer. Total mount. × 450. View showing late telophase. Note the connecting strand. The organism is heavily stained due to enormous number of food vacuoles.

Fig. 4. *Amœba dubia* Schaeffer. Total mount. × 450. View showing one method of cytoplasmic division in *A. dubia*. See text.

Fig. 5. *Amœba dubia* Schaeffer. Total mount. × 450. A later view of the same method of division as shown in Fig. 4. Note heavy connecting cytoplasmic strand.

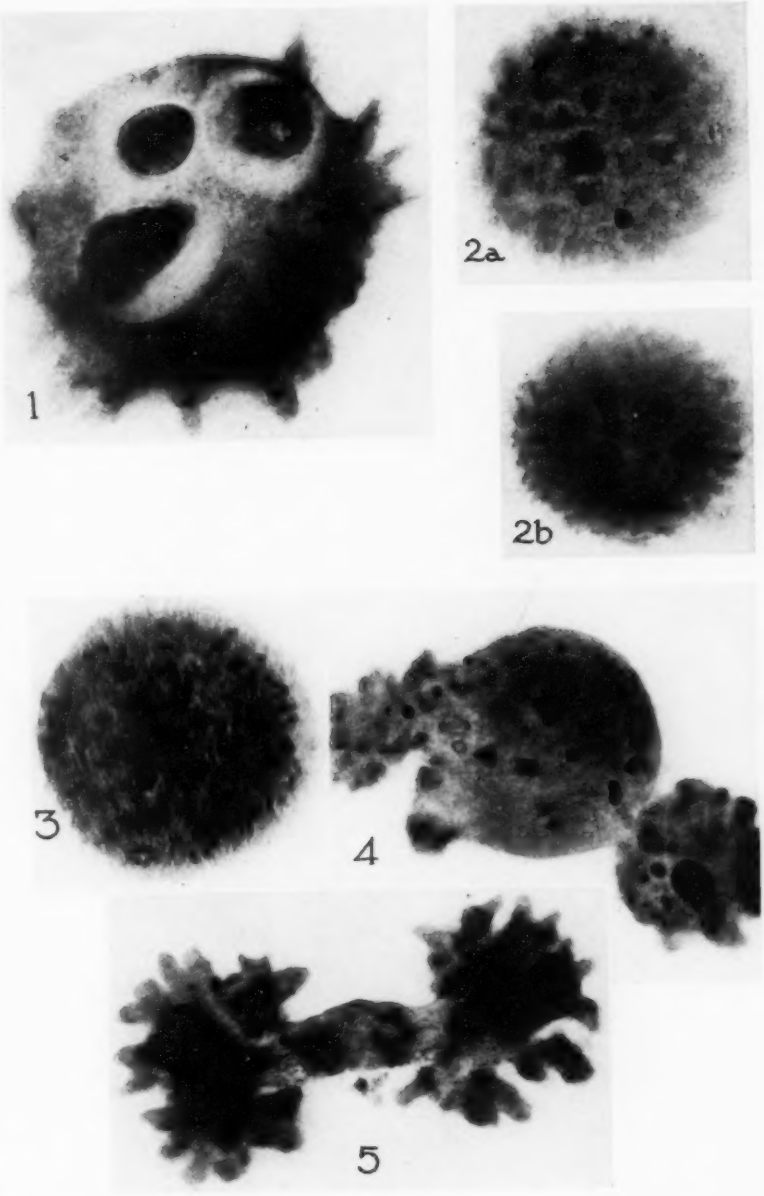

PLATE I

tinues, the sphere becomes slightly elongated. This elongation is a definite indication of the late telophase. From here on the external form of the dividing amœba may vary. In about 75 per cent of all cases, short, irregular pseudopodia break out at each pole. The remaining portion of the peripheral protoplasm of the former division sphere (plasmalemma and plasmagel of Mast) remains intact and no pseudopodia are projected from this region. A progressive flowing out occurs at each end and the pseudopodia of each prospective daughter amœba become longer and slightly thinner, still, however, remaining noticeably thickened. The cytoplasmic bridge gradually becomes more attenuated, finally forming a fairly long connecting strand (Plate I, Fig. 5). Cytoplasmic currents, as evidenced by the motion of crystals, can be observed to flow, first in one direction and then in the other in

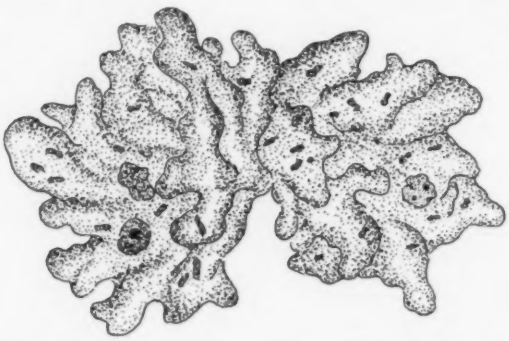

FIG. 6. *Amœba dubia* Schaeffer. Total mount. × 450. A view of the second type of division in *A. dubia*. See text.

this connecting strand which now appears quite homogeneous and clear. Just before this strand breaks all motion of protoplasm ceases. Each broken end snaps back toward its respective daughter amœba, and, becoming broader and thicker, is gradually withdrawn.

In other, fewer cases under apparently similar conditions no long connecting cytoplasmic strand can be seen. Here the pseudopodia are coarser and fewer in number and seem to flow in all directions. Unlike the former type, they are projected from the original central portion which makes the formation of a long connecting strand impossible. Instead, two wedge-shaped areas progressively pass through and cut off the daughter amœbæ (Fig. 6). In such cases the pseudopodia of the daughter amœbæ usually obscure the process of fission and it is only possible to know that it has been completed when the daughter cells move away.

Although the amœba while in the division sphere shows no signs of external motion, a condition of great internal activity prevails. Due largely to the spherical form and to the fact that *Amœba dubia* just before division (*cf.* Fig. 3) may ingest food actively, thus filling itself with many food vacuoles, it has been impossible to see the nucleus during fission. The cytoplasm, however, may be observed. It is in a state of extreme activity. There seems to be no phase difference between endo- and ectoplasm. Crystals and other inclusions are moved actively through the cytoplasm. In this 'boiling' motion the contained bodies appear to be shot against the plasma membrane and to rebound from it.

The Prophase

In *Amœba dubia* the vegetative or 'resting' nucleus consists of a nuclear membrane surrounding several thousand granules uniformly distributed through an achromatic matrix. These granules stain deeply with Heidenhain's hematoxylin and faintly with Feulgen stain. These we consider to contain the chromatin. From the vegetative condition to the prophase a disintegration or breaking down of the chromatin granules occurs. The earliest division phase shows a uniform, lightly staining matrix in which no fibers are evident. In this phase there now appear, peripherally arranged, a considerable number of larger achromatic bodies. The size of these varies from 0.5 μ to 4.0 μ and in the earliest phase we have counted as many as 50 in a single nucleus (Figs. 8 and 27). We have not, however, been able to determine with certainty the origin of these bodies.

In the later prophase, fibers may be seen. These fibers at first seem to run somewhat obliquely to the larger equatorial plane of the nucleus. The nuclear membrane remains intact and in appearance does not differ from the condition found in the vegetative nucleus. The cytoplasm immediately surrounding the nucleus is quite clear in all preparations. No difference can be detected in its appearance at this stage and in the 'resting' condition. The prophase lasts for approximately five minutes at a room temperature of 22° C. In this respect it is quite similar to the prophase of *A. proteus* as determined by Chalkley and Daniel. Due to lack of specific indicative features, it is difficult to obtain other than approximate time for this phase.

Metaphase

Although several preparations of the early metaphase were obtained, no definite equatorial plate as shown by Chalkley and Daniel for *A. proteus* could be observed. This we believe to be due to the fact that

such a plate obviously lasts for but a very short time interval. At this time also the chromatin stains very faintly. The nuclear membrane remains intact during this phase. In this respect *A. dubia* differs markedly from *A. proteus* in which according to Doflein (1913) and Chalkley and Daniel (1933) the nuclear membrane breaks down. The nucleus now takes a different shape, becoming elongated along the line of its shorter axis. The peripheral achromatic bodies begin to assume positions at the slightly invaginated poles. The spindle fibers fill the nucleus completely although they appear somewhat fainter in the polar regions (Fig. 28). The individual fibers are extremely fine, the number easily exceeds one hundred.

In the late metaphase the chromatin (chromosomes) now forms two well marked plates approximately 2.5 to 3.5 μ in thickness. The chromosomes are so numerous and small that we have been unable to identify single individuals.

Anaphase

In the early anaphase the nuclear form may best be described by calling it a 'barrel.' The spindle fibers fill the barrel running from top to bottom. The outer surface of the barrel is the nuclear membrane (Figs. 2*a*, 9, 10, 23, 29, and 30) which is slightly depressed or wrinkled

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Explanation of Plate II

Photomicrographs. All figures were photographed using a Leitz compound microscope and Leitz apochromatic lenses. Oil immersion lens, N.A. 1.32 and 15 × ocular were used in all cases except in Fig. 7 which was taken with a 5 mm. lens. Figures 11 and 17 were stained with Gentian Violet. Figure 12 with Picrocarmine. All others were stained with Heidenhain's hematoxylin. All figures are magnified 1350 diameters except Fig. 7.

Fig. 7. Vegetative nucleus showing dispersed chromatin granules. × 450.

Fig. 8. Prophase. Note large achromatic bodies and lack of chromatin granules. Same specimen drawn in Fig. 27.

Fig. 9. Anaphase (early barrel stage). Completely formed achromatic figure. The same nucleus is drawn in Fig. 29.

Fig. 10. Slightly later stage of anaphase. The same figure is drawn in Fig. 30.

Fig. 11. Late anaphase. The further separation of the chromatin plates is shown. Note also polar aggregation of achromatic bodies.

Fig. 12. Early telophase. Compare with Fig. 31.

Fig. 13. *a*. Stage similar to that in Fig. 12. The nucleus to the right is in sharper focus. Note chromatin plate. *b*. Same specimen showing left nucleus and spindle in sharp focus. Note numerous spindle fibers.

Fig. 14. Slightly later telophase.

Fig. 15. Telophase. Note beginning of 'dumb-bell' appearance and hemispherical daughter nuclei.

Figs. 16 and 17. Telophases showing further separation of prospective daughter nuclei. Note twisting of spindle. Hyaline polar zones shown in Fig. 17.

Fig. 18. Telophase showing daughter nuclei in two views.

Fig. 19. Late telophase showing 'thread' and ameboid daughter nuclei.

Fig. 20. Similar to Fig. 19 but somewhat later stage.

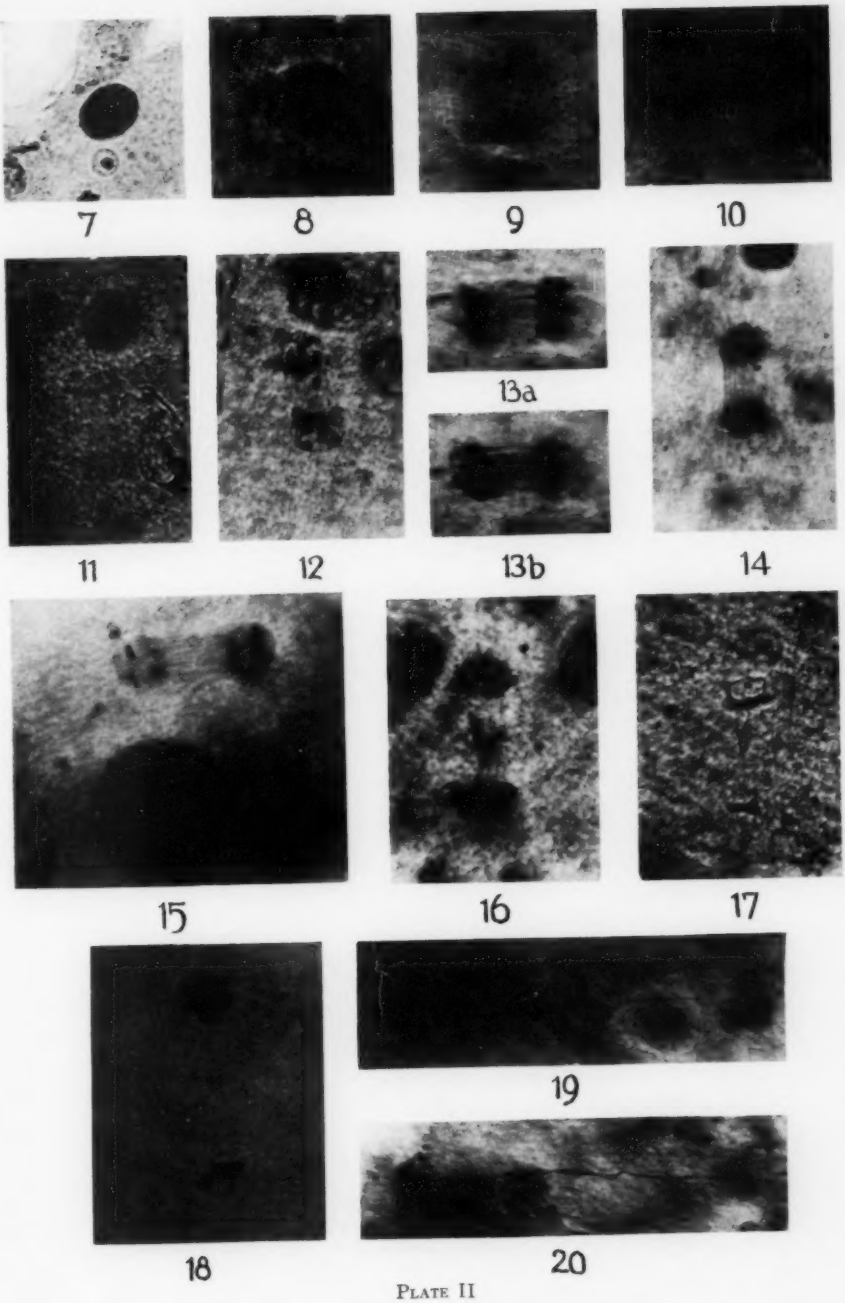

PLATE II

at each pole. From earlier to later stages of this phase the distance between the chromatin plates increases. In the late anaphase the majority of the peripheral achromatic bodies have assumed a polar position, i.e., between the chromatin plate and the pole.

Telophase

In the early telophase the barrel shape changes into that of a 'dumb-bell' with a very thick handle. The somewhat flat and more or less indented polar regions of the barrel now assume a hemispherical form, still containing practically all of the achromatic bodies. The spindle fibers extend from pole to pole and, in some preparations, appear to pass through the nuclear membrane which is attenuated in these regions. The chromatin plates occupy a position at each end of the handle of the dumb-bell, i.e., where the handle joins each rounded part (Figs. 2*b*, 11, 12, 13, 14, 15, 16, 31, and 32).

As the telophase progresses the prospective daughter nuclei gradually move farther apart and the distal portion of each appears distinctly hyaline. The achromatic bodies, which now have clearly decreased in number, are aggregated in these hyaline zones (Figs. 17, 18, and 33). The chromatin plates which in the early telophase were clearly defined now begin to lose this character and become dispersed through each daughter nucleus, obliterating the hyaline areas in each. With the further separation of the daughter nuclei, the spindle fibers become noticeably fewer in number. Almost invariably a twisting of the spindle cylinder occurs at this stage (Figs. 16, 17, and 32).

In the late telophase the outstanding feature is the transition of the elongated, twisted spindle cylinder to a long, thick, deeply-staining

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Explanation of Plate III

Photomicrographs. All magnifications × 1350. With the exception of Figs. 26 and 27 all figures are from sectioned amœbæ. Sections 5 μ thick. Fig. 25 from specimen stained with picrocarmine, all others stained with Heidenhain's hematoxylin.

Fig. 21, *a–g*. Views of serial sections, complete, of a typical 'resting' nucleus of *Amœba dubia*. Fig. 21 shows the entire section of the amœba, the nucleus of which is again represented in Fig. 21*e*. Note the uniform distribution of the chromatin granules in all sections of the nucleus.

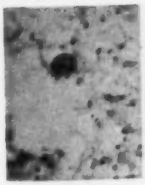
Fig. 22 *a*, *b*, and *c*. Successive sections through an early metaphase. Sectioned obliquely.

Fig. 23. Late anaphase. Median longitudinal section. Note achromatic figure.

Fig. 24. Late telophase showing ameboid nucleus and part of thread. Nucleus staining heavily in this stage.

Fig. 25. Total mount. Reconstructing nucleus soon after fission. Note presence of large achromatic bodies and reappearing chromatin granules.

Fig. 26. Reconstructing nucleus about two and one half hours after fission. Same nucleus as drawn in Fig. 36.



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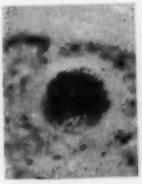

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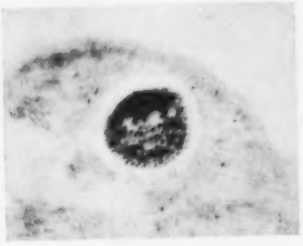

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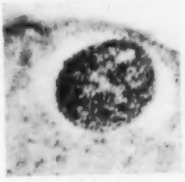

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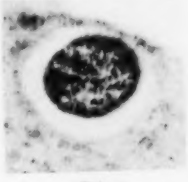

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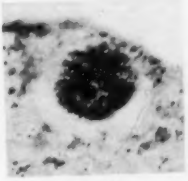

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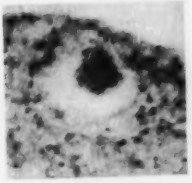

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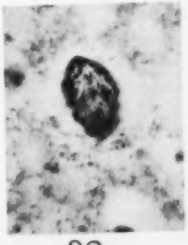

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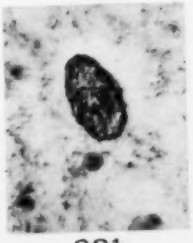

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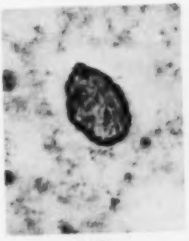

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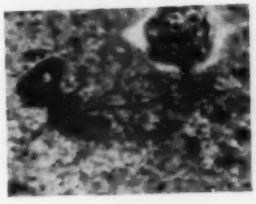

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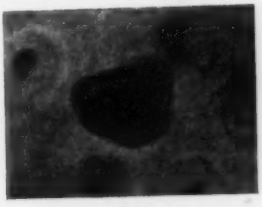

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PLATE III

strand or thread. That the major part of this strand is formed by the nuclear membrane is impossible to doubt since it can be followed completely around each daughter nucleus. At the place where the strand passes into the nucleus, a conical, vacuolated region may be seen (Fig. 34). The form of each daughter nucleus gradually changes from the roughly hemispherical condition in the dumb-bell stage to that of one which is quite ameboid in appearance (Figs. 19, 20, 24, 33, and 34). The daughter nuclei occupy a position at opposite ends of the now elongated mother amœba. They are extremely close to the periphery. Each daughter nucleus now stains much more heavily throughout. The chromatin appears as very small, staining granules. These granules increase rapidly in size and are quite noticeable at the moment of fission. They are quite uniform in size, measuring approximately 0.2 μ in diameter. The telophase nuclei show a striking contrast in apparent size. This is due to the fact that one of the nuclei is so oriented as to show a surface view while the other is seen from the side (Fig. 18). The conspicuous fiber formed from the nuclear membrane breaks, so far as ascertained, apparently shortly after the cytoplasmic separation has begun. In some cases we have found that this break occurs close to one of the nuclei. We have no evidence that it is reabsorbed since no stages in that process have been found in many preparations made at

EXPLANATION OF PLATE IV

All drawings made with camera lucida. × 2100.

FIG. 27. Prophase (same as Fig. 8). Chromatin granules of vegetative nucleus have disintegrated. Large achromatic bodies clearly defined in lightly staining nuclear matrix. Note slight invaginations at poles.

FIG. 28. Slightly later stage. Formation of achromatic figure is completed. Slight elongation of nucleus has taken place and polar invaginations are more prominent.

FIG. 29. Late metaphase. Illustrates typical 'barrel' stage. Note chromatin plates staining faintly and polar aggregation of achromatic bodies. Nuclear membrane clearly marked (same as Fig. 9).

FIG. 30. Anaphase (late). Here chromatin plates stain more heavily and the distance between the plates has increased (same as Fig. 10).

FIG. 31. Early telophase. Note beginning of 'dumb-bell' with thick handle, and hemispherical form of polar regions containing most of the achromatic bodies (same as Fig. 12).

FIG. 32. Later telophase. Chromatin plates are not so pronounced. Typical twisting of spindle cylinder shown (same as Fig. 16).

FIG. 33. Still later telophase. Reduction in number of spindle fibers. Daughter nuclei assuming typical ameboid shape. Reduction in number of achromatic granules. Entire nucleus staining more deeply.

FIG. 34. Latest telophase. Note characteristic 'thread.'

FIG. 35. Showing nucleus just after fission is completed. Note presence of numerous fine granules and dispersion of achromatic bodies throughout the ameboid nucleus.

FIG. 36. Reconstructing nucleus two and a half hours after fission. Note increase in size of nucleus and of chromatin granules. Achromatic bodies still present. Nucleus more regular in outline (same as Fig. 26).

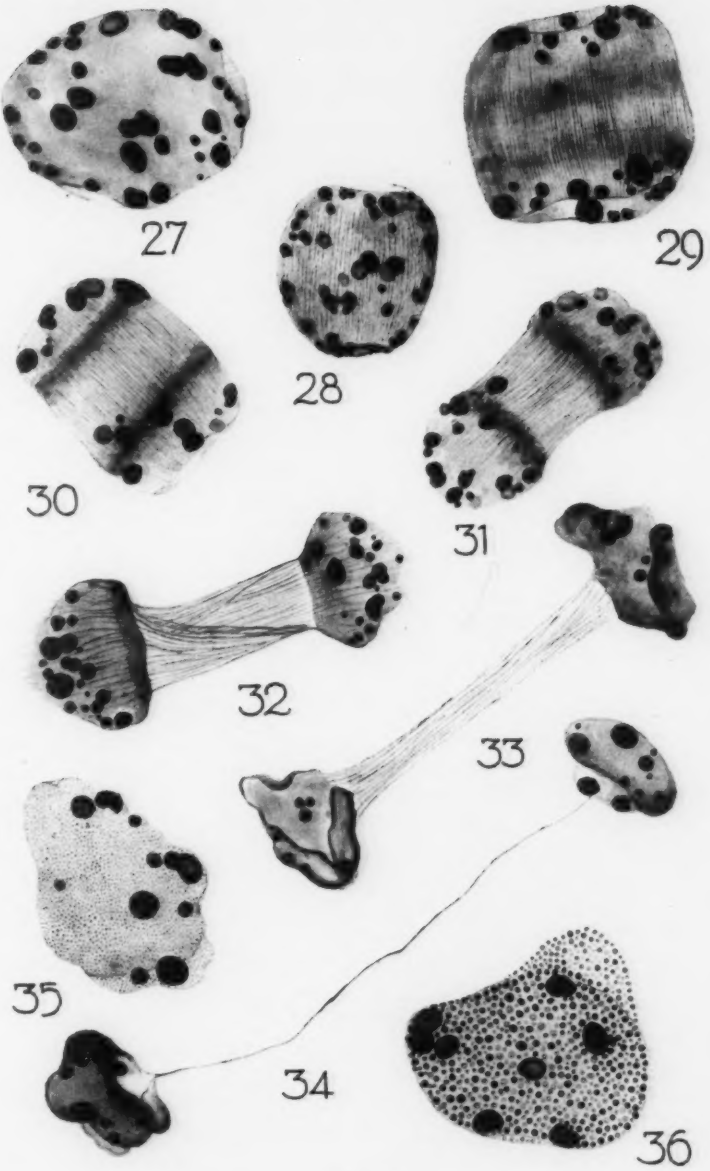

PLATE IV

this time. No remnants of it ever appear in newly divided daughter cells. We infer that it is resorbed by the cytoplasm.

Reconstructing Nuclei

The characteristic appearance of the newly separated nucleus is like that of a small amœba with very blunt pseudopodia. This nuclear configuration not only appears in all stained preparations of this stage but also may be seen in the newly divided daughter amœbæ (Fig. 35). In the early reconstructing nucleus the achromatic bodies are present but few in number (Figs. 25 and 35). A few minutes after division the chromatin is present in the form of very numerous, staining granules slightly smaller than those found in the typical resting nucleus. The picture shown by the reconstructing nucleus is as follows—within a varying period (three to five hours) the outline of the nucleus becomes regular like that of the ellipsoidal vegetative nucleus. During this time the achromatic bodies gradually disappear. They vary considerably in size up to their final disappearance in the typical resting nucleus. Figures 26 and 36 show the same reconstructing nucleus two and a half hours after fission. At this time the size of the chromatin granules is 0.8 μ in diameter.

Discussion

In the present study of mitotic division in *Amœba dubia* a number of significant differences between this species and other free-living amœbæ have been found. We believe that these variations depend upon fundamental structural differences in the nucleus. It has already been shown that the nucleus of *Amœba dubia* does not conform with any of the types of nuclei described by Schaeffer (1926) in his comprehensive review of the biology of amœba. In complete studies of the process by numerous investigators, e.g., Gläser (1912), Dobell (1914), Wasielewski and Kühn (1914), Jollos (1917), Doflein (1912) and others, the nucleus is invariably described as possessing an endosome or karyosome. In the nucleus of *Amœba dubia* no endosome exists but the chromatin is evenly distributed throughout the entire ' resting ' nucleus.

One of the outstanding features is that the nuclear membrane remains intact throughout the entire process. In the closely related species, *A. proteus* (Chalkley and Daniel, 1933) the nuclear membrane entirely disappears during the early anaphase. In general this is in accord with the findings of the above-mentioned investigators with the exception of Gläser. It would seem clear from our study that both chromatic and achromatic elements of the spindle are derived solely from the nucleus.

A second interesting feature is the presence of the large achromatic bodies to which we have so often referred. In our preparations we have used a considerable variety of both fixatives and stains. These bodies do not stain with any chromatin stains. As previously stated, these appear in large numbers in the early prophase, they are present throughout the entire process of fission and gradually disappear as the nucleus is reconstructing. There may be a correlation between the disappearance of these achromatic bodies and the increase in size of the chromatin granules in such nuclei. At present we are unable to offer an explanation of the constitution and function of these bodies.

The mitotic process in *Amœba dubia* as shown by this study shows striking differences to that of *Amœba proteus* and thus supports the view that these two common, large amœbæ are quite distinct species.

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THE CHROMOSOME CYCLE OF PHENACOCCUS (COCCIDÆ)

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Within the insect family Coccidæ are species in which male haploidy is completely established and others in which a physiological haploidy seems to be in progress of evolution through the inactivation of one of the haploid sets of chromosomes in the male line. To the latter group belongs *Phenacoccus acericola.* In general its cytology conforms closely to the *Pseudococcus* type (F. Schrader, 1921, 1923); but the final stages of spermatogenesis are fortunately more open to analysis and the complete loss of those spermatid nuclei which receive the degenerate haploid contingent of chromosomes can here be established without a doubt. The case is thus also closely allied to that of *Gossyparia* (F. Schrader, 1929), but it is not complicated by the peculiar pairing among the degenerate chromosomes which partially obscures the interpretation of events in the latter species. These general conclusions on *Phenacoccus* were tentatively expressed in a review of coccid cytology in relation to haploidy (F. Schrader and Hughes-Schrader, 1931); the data demonstrating them are now complete and it seems wise to present the proof in brief form in order to complete the record.

General Biological Features

The sugar maple is the only known host of *Phenacoccus acericola.* Infections are sporadic and of rather rare occurrence. My material comes from a small colony on a single tree in the Arnold Arboretum, Boston, and a larger colony infesting a group of trees in the depot park at Wellesley, Massachusetts. I am indebted to Dr. Harold Morrison of the U. S. Bureau of Entomology for the discovery and identification of the first colony, and to Dr. M. A. Hayden of Wellesley College for continued assistance in connection with the latter.

Only a single generation is produced annually. Adult males appear late in August and early September. The fertilized females hibernate in crevices of the bark, emerging early the following summer and attaching themselves to the main veins on the under side of the leaves. The eggs are laid in a loose wax mass adherent to the body of the female;

on hatching the larvæ crawl out and attach to the smaller veins of the leaves. At the start of the third instar the male larvæ spin characteristic wax cocoons in which they complete the two final molts. These cocoons are usually hidden away in twig axils or bark crevices.

Meiosis in the male occurs in the third instar, with spermatid development carrying over into the fourth. The eggs develop slowly throughout the summer, maturation and fertilization occurring during the early fourth instar. The eggs are fertilized in the oviducts and development proceeds to varying stages of embryology before the eggs are laid. No evidence of parthenogenesis was found, but this has not been checked by adequate breeding experiments.

Best fixation was obtained with Allen's modification of Bouin's fluid, after a complete dissection of ovaries and testes in the fixative. The eggs must be punctured to insure penetration. Ovaries were sectioned at 5 and 6 micra, testes at 3 micra,—as the cells are extremely small. The preparations illustrated were all stained with the Haidenhain iron hematoxylin method.

Cytological Observations

The diploid chromosome number in both sexes is twelve. The cytology of the female is normal in every respect. The eggs undergo two maturation divisions with typical synapsis, tetrad formation, and reduction of the chromosomes to the haploid set of six.

As indicated above, the interest of the present case lies in the chromosome cycle of the male line. Here one whole haploid group of chromosomes maintains a permanently condensed form, massing into a pycnotic knot during the resting phases, and separating into its component chromosomal elements for each division. Somatic and spermatogonial cells all show this permanent pycnosis of one chromosome set.

The prophase of the primary spermatocyte is initiated by the gradual separation of the pycnotic mass into its six component chromosomes (Figs. 1, 2, and 3). Only then do the normal chromosomes emerge from the chromatin reticulum as fine threads which shorten, thicken, develop each an equational split (Figs. 4 and 5), and finally condense into the small rod-shaped chromosomes of the metaphase stage (Fig. 6). The permanently condensed group is distinguishable for some time by its close aggregation, the normal chromosomes usually surrounding those of pycnotic origin rather loosely (Fig. 6); with the completion of the metaphase plate, however, this distinction is lost (Fig. 7). The first division proceeds as a normal mitosis; a typical bipolar spindle is formed and each chromosome divides equationally (Figs. 8 and 9). Early in the anaphasic movement the chromosomes of each daughter group segre-

gate once more into two groups; one set of six adheres closely together, often in ring formation, and retains the compact form and densely staining aspect of the metaphase chromosomes; the other six show no tendency to aggregate and stain more lightly. In Figs. 10, 11, and 12 the ring-shaped group of six is seen from its side; the polar aspect of the telophase group (Fig. 13) shows a top view of the ring, surrounded by the six loosely arranged chromosomes. No ordinary interphase occurs; the telophase configuration of the first division becomes the metaphase for the second with no rearrangement in position nor change in form of the constituent chromosomes.

From the ring of six condensed chromosomes a half spindle now forms, converging in a single centriole (Fig. 14). The normal chromosomes show no trace of division apparatus other than such an occasional weft or fibril as is shown on one chromosome in Fig. 15. Indeed, from their increasingly indefinite outline, the chromosomes of the normal

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PLATE I

Drawings with Abbé camera lucida, at table level; Zeiss 2 mm. obj. and 20 × ocular.

FIG. 1. Resting phase of pre-meiotic spermatogonium; permanently condensed chromosomes in pycnotic knot.

FIGS. 2 and 3. Prophases of primary spermatocytes with condensed chromosomes separating from knot.

FIG. 4. Normal chromosomes evolving as threads from the reticulum in later prophase I.

FIG. 5. Later stage of prophase I. Normal haploid set of chromosomes showing equational split.

FIG. 6. Metaphase I with two haploid groups still spatially distinct.

FIG. 7. Later metaphase I; no distinction between the haploid groups.

FIGS. 8 and 9. Lateral views of anaphase I.

FIGS. 10, 11, and 12. Later anaphases of first division. Cytoplasmic stalks visible. In each anaphase group the six chromosomes derived from the condensed set form a ring, here viewed from its side.

FIG. 13. Polar view of telophase I, with the two sets of chromosomes distinguishable.

FIGS. 14 and 15. Formation of a single half spindle in connection with the condensed chromosome group. Normal group passive.

FIGS. 16 and 17. Anaphasic movement of the condensed group to the single pole. Interzonal body forms between the two groups.

FIG. 18. Telophase II, with six chromosomes in each group. No cytoplasmic division.

FIG. 19. Binucleate spermatids; growth of the normal nucleus.

FIG. 20. Fusion of spermatids in pairs, forming quadrinucleate spermatids, each with two normal and two abnormal nuclei.

FIGS. 21, 22, 23, 24, and 25. Development of sperm from the normal nuclei; gradual degeneration of the abnormal nuclei.

FIG. 26. The abnormal nuclei reduced to pycnotic knots and sloughed off with the cytoplasmic residue of the cells. Mature sperm forming bundle at edge of cyst.

PLATE I

group would seem to be entering an interphasic rest. The condensed group now moves poleward, away from the loose group,—its half spindle shortening and the cell as a whole elongating in the process (Figs. 16 and 17). Between the anaphasic groups a definite interzonal body forms, quite similar in form and staining reaction to those of orthodox mitoses. For a short time the chromosomes are countable at both poles—six in the massed, compact group, six in the looser group. They quickly assume the resting condition and nuclear membranes form around them. No trace of a cytoplasmic division occurs, and all the spermatids are thus at first binucleate.

The binucleate spermatids now fuse in pairs. Some direct observational evidence for this lies in the frequently lobed form of the early quadrinucleate spermatids resulting from the fusion. It seems probable that the first division, although apparently complete cytoplasmically, does not cut through the short centrally directed stalk which each cell of a cyst shows when cut radially (Figs. 9, 10, 11, and 18). The spindle of the first division lies always at right angles to this stalk; thus a division of the cell, apparently complete when viewed from the outside of the cyst, would not cut this stalk (Fig. 12). The refusion of the cells may well proceed from these persistent stalks. A further check on the fusion of the spermatids is available in counts of the number of nuclei per cyst in the affected stages—a method which has proved useful in the study of several other coccids. Cysts of primary spermatocytes contain typically sixteen cells; those with secondary spermatocytes thirty-two. The binucleate spermatids show the same thirty-two cells, but sixty-four nuclei per cyst. After fusion, the cysts of quadrinucleate spermatids contain the sixty-four nuclei expected.[1]

The further history of the spermatid development can be read directly from Figs. 19 to 25 without much comment. The two small darkly-staining nuclei derived from the condensed group of chromosomes show almost no growth, and gradually degenerate *in situ* into crumpled pycnotic knots. From the two more slowly evolving, normal nuclei in

[1] The following counts establish the uniformity of the number of nuclei per cyst, and give evidence of the fusion in pairs of the spermatids.

Number of nuclei per cyst of primary spermatocytes: 16—16—12—16—16—16—3—16—15—16—16—16.

Number of nuclei per cyst of secondary spermatocytes: 32—32—32—31—31—32—27—32—31—24.

Number of nuclei per cyst of binucleate spermatids: 64—64—62—65—61—64—65—68—44—25—48.

Number of nuclei per cyst of quadrinucleate spermatids: 64—62—57—67.

Number of sperm per cyst bundle: 32—33—28—31—34—31—30—28—28—26.

Number of pycnotic nuclei per cyst after sperm formation: 32—31—32.

each spermatid typical coccid sperm develop (Figs. 19 to 24). The sperm formed in each cyst then coil into a spiral bundle; cross sections of these bundles, although too small and closely packed for accurate counts, show approximately thirty-two sperm per bundle.[1] The degenerate nuclei, sloughed off by the sperm with the cytoplasmic residue of the cell (Fig. 25), are also countable for some time; again the number per cyst is consistently thirty-two,[2] confirming the complete loss of that half of the spermatid nuclei derived from the pycnotic chromosome set.

The degenerate condition of one of the haploid chromosome sets in the male expresses itself somatically as well as in the meiosis of the germ cells. In all the body cells of males the affected chromosomes retain the condensed form throughout the resting phases, mass into a pycnotic knot, and separate into their component elements only at each nuclear division. As in *Gossyparia*, this behavior first becomes visible in young embryos of the blastula stage.

Summary

1. The diploid chromosome number of *Phenacoccus acericola* is twelve in both sexes.

2. The female is normal in all respects. All eggs undergo two maturation divisions with typical synapsis and reduction of the chromosomes to the haploid condition.

3. In the male, from the blastula stage of development on, one haploid set of chromosomes maintains a permanently condensed condition, forming a pycnotic knot in resting phases and separating into its component chromosomes at division.

4. Meiosis in the male is effected without synapsis. The first division is equational and diploid. Without interphase the telophase chromosomes separate into two groups of six, one condensed and closely aggregated, and one loosely arranged and more diffuse. A single half spindle forms in connection with the condensed group and reduction is effected by the consequent passage of the condensed group to the single pole of the spindle.

5. No cytoplasmic division accompanies the second division; the binucleate spermatids thus formed fuse in pairs.

6. That half of the spermatid nuclei originating from the condensed chromosome set disintegrates without trace of sperm development; the other half of the spermatid nuclei, those originating from the non-pycnotic normal chromosomes, develops into typical coccid sperm.

[2] See footnote 1.

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